Shpol'skii spectroscopy and synchronous fluorescence spectroscopy

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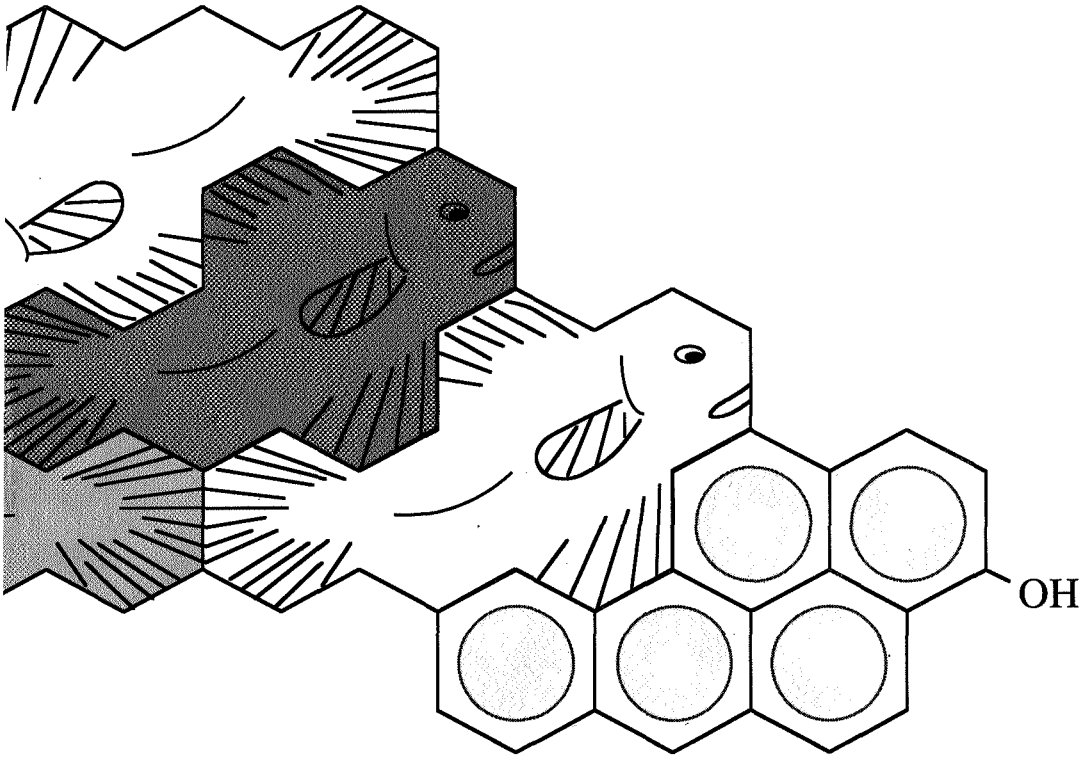
SHPOL'SKII SPECTROSCOPY AND SYNCHRONOUS FLUORESCENCE SPECTROSCOPY

(BIO)MONITORING OF POLYCYCLIC AROMATIC HYDROCARBONS AND THEIR METABOLITES



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VRIJE UNIVERSITEIT

SHPOL'SKII SPECTROSCOPY AND SYNCHRONOUS FLUORESCENCE SPECTROSCOPY:

(BIO)MONITORING OF POLYCYCLIC AROMATIC HYDROCARBONS AND THEIR METABOLITES

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ten overstaan van de promotiecommissie
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aan allen
die mij dierbaar zijn

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1
GENERAL INTRODUCTION

POLYCYCLIC AROMATIC HYDROCARBONS AS ENVIRONMENTAL CONTAMINANTS

Sources and distribution

Polycyclic aromatic hydrocarbons (PAHs) are usually defined as a group of chemicals consisting of two or more fused benzenoid rings and containing no other elements than hydrogen and carbon. Sometimes the term polycyclic aromatic compounds (PACs) is used to explicitly include aromatic compounds containing nitrogen, oxygen or sulfur heterocycles or substituents, as these often possess similar chemical and ecotoxicological properties. Molecular structures of selected PAHs are given in Fig. 1.

PAHs are natural constituents of crude oil and many other petrochemical products. These aromatic structures have been formed during the course of millions of years by successive dehydrogenation of organic material at relatively low temperatures. In PAH mixtures of petrochemical origin 2- or 3-ring compounds, such as naphthalene, phenanthrene, and their respective alkylated derivatives, are more abundant than the heavier PAHs containing 4 or more rings.

PAHs can also be formed at more elevated temperatures during the inefficient combustion of fossil fuels or other organic matter. A typical PAH mixture of a high-temperature combustion source contains mainly unsubstituted compounds, and the PAHs of 4 and more rings are more abundant than the smaller ones. PAHs in natural samples are always encountered as mixtures: the relative distribution of the various PAH congeners, the PAH "profile", provides a clue to the origin (petrochemical or pyrolytic) of the pollution. (Bouloubassi and Saliot, 1991).

Natural sources of PAH emission have always existed (sediment erosion, volcanic eruptions, forest fires), but since the industrial revolution, there has been a rapid increase in the loading of the environment with petrochemical PAHs (oil spills) and particularly with PAHs of pyrolytic origin. Spectroscopic determination of combustion-related PAHs and their biotransformation products (metabolites) will be the subject of this thesis.

Sometimes, the connection between the formation of PAHs and the absorption by an organism is quite straightforward, as in the case of people smoking tobacco, but for relatively persistent chemicals like PAHs more complex routes of distribution and uptake are also possible. PAH molecules in automobile exhaust (gaseous or aerosol-bound), may be transported through the atmosphere, be deposited on trees, be washed down with the rain into the river, and finally end up in a sedimentation sink or enter the aquatic food chain. Creosote, a distillation fraction of coal tar, is widely used as a wood preserving agent. PAHs leaking into the water from creosoted pilings may accumulate in mussels (Dunn and Stich, 1976), and thus be passed on to lovers of seafood. These examples merely serve to illustrate that the distribution patterns of PAHs in our environment can be extremely complex, and will also depend on vapor pressure, aqueous

solubility, or chemical/biological inertness of the particular congener. Various physical, meteorological, hydrodynamical, chemical, biological and even economical factors need to be considered in order to get insight into the complete mass balance. Only then we can link environmental exposure levels and possible ecotoxicological effects to major sources. In this respect, reliable chemical analysis of PAHs and their biotransformation products in the various environmental compartments is of crucial importance.

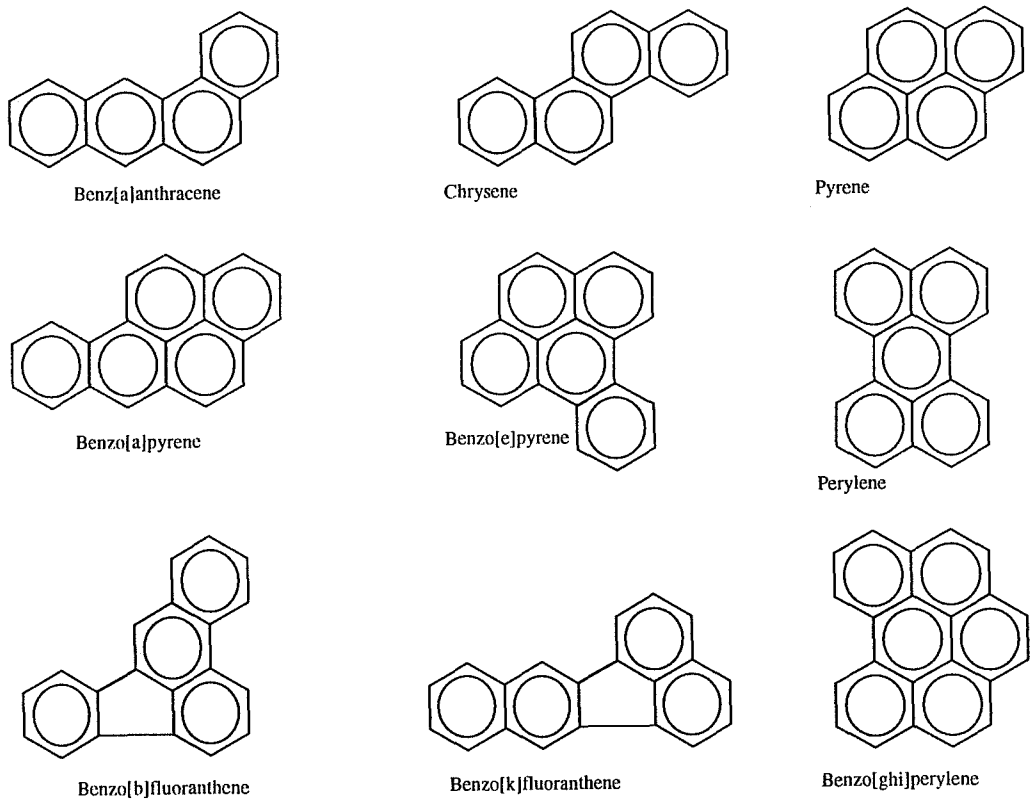


Fig.1 Molecular structures of selected PAHs.

Biological effects

Contrary to "modern" contaminants, such as pesticides or PCBs, human exposure to PAHs has probably started far back in the prehistoric age with the use of fires for cooking and heating. Already in 1775 the English surgeon Pott noticed an unusually high incidence of scrotum cancer in chimney sweeps and postulated a connection with occupational exposure to soot. Large-scale epidemiological surveys revealed a statistically significant enhancement in lung cancer mortality among top-side cokeoven workers in the United States (Lloyd, 1971). The high incidence of lung cancer in a rural area in the Chinese province Xuan Wei, in particular among non-smoking women, was thought to be connected with the use of "smoky coal" for cooking (Chuang et al., 1992).

Experimental evidence for the carcinogenic properties of coal tar was presented in 1915 by Yamagiwa and Ichikawa, who succeeded in inducing tumors by the application of coal tar to the ears of rabbits. In the early 1930s several PAHs were isolated from coal tar and separately tested for carcinogenic activity. One of these pure compounds, a 5-ring aromatic hydrocarbon, was found to be extremely potent. This compound is now known as benzo[a]pyrene, BaP (see Phillips, 1983, for a historical overview on BaP research). At present, numerous PAHs or PAH derivatives are listed as proven or suspected carcinogens (see Karcher et al., 1983, 1988, 1991, for references). Grimmer and coworkers (1991) reported that, after HPLC fractionation of automobile exhaust particulate matter, 81 % of the total carcinogenic activity of the mixture could be attributed to the PAH-fraction; BaP accounted for 2.4 % of the total activity. BaP is by far the best studied PAH as for carcinogenicity, and the BaP concentration in a sample is often used as an indicator for the total carcinogenic potency (Kramers and van der Heijden, 1988).

It has been known for some time that BaP itself is a relatively inert compound that needs to be metabolized before it can exert genotoxic activity. Many biotransformation pathways exist (Hall and Grover, 1990), of which some are depicted in Fig. 2 . Biotransformation starts with the binding of the xenobiotic compound to the cytochrome P450 enzyme system, which catalyzes the addition of an oxygen atom across a double bond of the molecule, thus forming an epoxide. This epoxide may subsequently be coupled to glutathion, isomerize into a phenol, or be hydrolyzed to yield a saturated dihydrodiol moiety. Phenols and dihydrodiols can be conjugated to glucuronic acid or sulfate to facilitate excretion. Overall, the biotransformation of the strongly lipophilic BaP results in the formation of more polar metabolites that are more easily excreted than the parent compound. The result of these detoxification mechanisms is that the bulk of PAH molecules, after absorption by higher species, is also quickly removed from the body (Chipman et al., 1982). However, some reactive intermediates, such as (+)-7R,8R-dihydrodiol-9S,10R-epoxide BAP (BPDE) may also be formed during the process. These intermediates have been shown to form stable adducts with proteins or with DNA, which could probably lead to tumor initiation if the defective nucleotide is not repaired in time. The formation of reactive dihydrodiol epoxides (ultimate carcinogens) has also been demonstrated for other PAHs containing an angular benzene ring, and is believed to be a general mechanism (Hall and Grover, 1990).

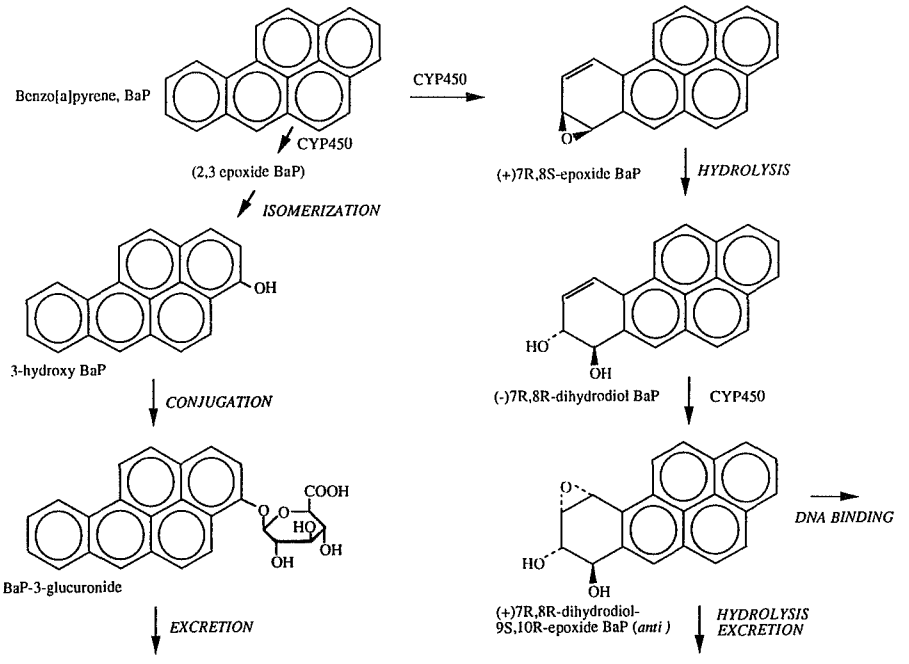


Fig. 2 **Selected metabolic pathways illustrating the detoxification vs. activation of benzo[a]pyrene. CYP450 = cytochrome P450.**

From the mechanism described above, it can be concluded that species which do not possess a well developed mixed-function oxygenase enzyme system are likely to accumulate parent PAHs. The elevated PAH body burdens in the tissues of such species are not likely to exert genotoxic effects (Dunn, 1991), but could lead to other pathological disturbances (Moore et al., 1989).

On the other hand, species which show a high level of metabolic activity, but lack an effective DNA repair mechanism, and also live long enough for tumors to develop, are probably most susceptible to PAH-induced carcinogenesis. PAH metabolism in fish liver is to a large extent comparable to that in mammals (Varanasi, 1986), but DNA repair is substantially less efficient (Walton et al., 1983). Fish populations with a high incidence of liver neoplasms have been found in several areas in the United States, for instance in the Black River, Ohio, in the vicinity of a coke plant (Baumann et al., 1987). Malins and coworkers (1988) reported that the incidence of liver neoplasia in English sole (*Parophrys vetulus*) at various sites in Puget Sound, WA, was highly correlated with local PAH pollution levels in the sediments, while the correlation with other suspected contaminants (PCBs, heavy metals) was much weaker. Vethaak (1992) carried out a number of large-scale fish disease surveys in the Dutch coastal area and arrived at a similar conclusion.

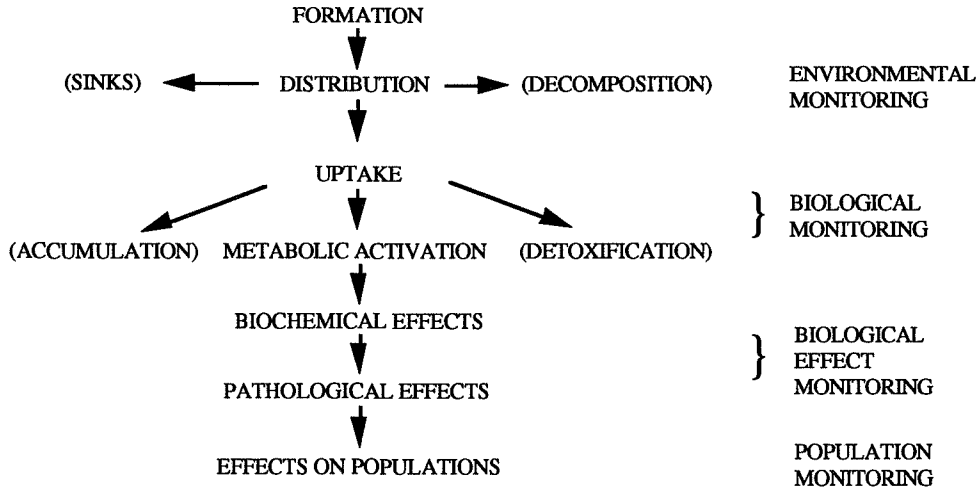
In response to these concerns, the sources, distribution, fates, and effects of PAHs in the aquatic environment are intensively studied. In chapters 4-6 we will describe the development of some innovative analytical approaches which can be applied to determine PAH pollution levels in the aquatic environment and the extent of uptake of these chemicals by aquatic species.

Evidently, also higher species with more efficient DNA repair mechanisms may still be at an elevated risk of developing cancer if they are regularly exposed to high levels of PAHs. The health risks associated with PAH exposure are of great concern in industrial toxicology. Chapter 7 describes an analytical procedure to assess the uptake of benzo[a]pyrene by cokeoven workers.

ASSESSMENT OF PAH EXPOSURE: ENVIRONMENTAL VS BIOLOGICAL MONITORING

Monitoring approaches

Schematically, the complex pathway of a typical PAH molecule from source to effect may be depicted in the following manner:



Analytical chemists can determine PAH levels at a specific location, while physicians and biologists can document cancer incidences in a particular population at the same site. Finding a statistically significant correlation, however, does not necessarily mean that PAH exposure has indeed produced the observed effects. Although the genotoxic properties of a number of PAHs have been convincingly demonstrated in various laboratory experiments, simply showing that these potentially toxic compounds are present in the environment is not sufficient, since exposure always occurs to a myriad of different substances simultaneously. Therefore, we can only attempt to obtain further circumstantial evidence to prove or disprove the assumed causal link. Studying the uptake of PAHs by the particular population, the extent of PAH metabolism, the occurrence of DNA damage or specific side-effects (e.g., enzyme induction), or the occurrence of pathological prestadia, may all contribute to fill the gap between environmental contaminant levels and epidemiological data.

In the case of PAH exposure, regular monitoring of PAH occurrence, uptake, and possible effects can be carried out at different levels. Analytical chemists can contribute little to purely biological disciplines like population monitoring, or the monitoring of pathological effects at the individual, organ, or cellular level. Chemical methods become more important at the biochemical level: the identification of PAH adducts to DNA or protein can serve as an indication that reactive intermediates of a particular PAH have been formed. Enhanced activity of the cytochrome P450 enzyme system can serve as a more general indication of recent exposure to foreign chemicals (but not specifically PAHs). The systematic study of these or related effects is generally called biological effect monitoring (BEM). Once a suitable biological or biochemical effect parameter has been found, it can serve as an "early warning signal", so that potential risks can be identified at an early stage and measures be taken before irreversible effects would occur.

At an even more basic molecular level, it would be useful to determine the amount of a given PAH that has actually entered the body as the result of exposure to a PAH-containing environment. Depending on the metabolizing capacities of the particular organism, the uptake rate can be assessed by measuring either the parent compound or the biotransformation products. This type of activity, that focuses on the determination of the actual internal dose, is commonly referred to as biological monitoring (BM).

Finally, it is of course important to carry out a systematic determination of PAHs in the various environmental compartments (environmental monitoring, EM). We may assume that the PAH uptake is related to the environmental levels, but that relationship can be a very complex one, given the numerous factors that may influence the rate of absorption, especially if several uptake routes are available. Environmental data are also required for the understanding of the distribution patterns of these chemicals, which is needed in order to trace back a possible effect to the source.

Presently available methods; environmental monitoring

PAH levels are regularly monitored in various types of samples, such as soils and sediments, water, outdoor and workplace air samples, and food products. The complete procedure involves collection of a sample or preferably a set of subsamples, pretreatment of the sample (which may include homogenization, extraction, one or several clean-up steps, and often trace enrichment), and finally the quantitation of selected individual compounds. Several techniques are available for the determination of PAHs in extracts from environmental samples. The methods most commonly applied are column liquid chromatography with fluorescence detection (HPLC-Flu), and gas chromatography with flame ionization or mass spectrometric detection (GC-FID; GC-MS). Intercomparison exercises have revealed that sometimes large systematic differences exist between analytical results produced by different laboratories or between data obtained with different methods. Difficulties associated with PAH analysis are photodecomposition, losses and random errors during the various sample treatment steps, overlap caused by incomplete separation, and non-linearity of response. A specific problem is the fact that with most techniques it is often not possible to distinguish between isomeric PAHs. Isomer-specificity is crucial since the carcinogenic activities of PAHs often varies markedly between isomers.

New analytical techniques that combine excellent sensitivity with a high degree of selectivity would be most welcome, especially if they do not require extensive cleanup, and if they show good isomer-specificity. In general, the availability of alternative techniques, that are based on different physico-chemical principles, is important for the quality assurance of analytical procedures.

Presently available methods; biological monitoring

As mentioned above, the uptake of PAHs from the environment may be quantitated by determination of either the parent PAH or the metabolites of that compound. The first approach can be used to study the uptake and accumulation of PAHs by organisms that show only marginal metabolic activity, such as mussels (Dunn and Stich, 1976; Boom, 1987). In rapidly metabolizing species, however, the steady-state PAH body burden will be very low (Varanasi et al., 1985; Van der Oost et al., 1993), and chemical analysis of parent PAHs, if relevant at all, would require very sensitive methods.

In such species, the PAH uptake rate or the PAH flux is more accurately determined by the measuring PAH metabolites in excreta (urine, faeces, or gall-bladder bile). In laboratory experiments, after the administration of a relatively high dose of a single PAH compound to a test animal, metabolites are often easily determined with HPLC. PAH metabolite analysis in real samples is far more difficult, owing to the simultaneous exposure in the field to a complex mixture of PAHs. Furthermore, PAH exposure levels in the field are often several orders of magnitude lower than the doses typically applied in laboratory experiments. Krahn et al. (1987) developed an HPLC method for the determination of selected (mainly smaller) PAH metabolites

in fish bile samples from the field, but the method was not always sufficiently sensitive for the detection of metabolites of heavier, more carcinogenic PAHs, such as benzo[a]pyrene. Also GC-MS (after chemical derivatization) proved only applicable to metabolites of small PAHs (Krahn et al., 1987).

In occupational toxicology, the determination of PAH metabolites in urine can be very useful as a monitoring tool to assess PAH exposure. Becher and Björseth (1983) proposed to reduce the urinary PAH metabolites back into the original parent compounds with strong acid, prior to HPLC analysis, but no details were presented as to the completeness and specificity of the method. Uziel and coworkers (1987) tested the utility of synchronous fluorescence spectrometry for the determination of 7,8,9,10 tetrahydroxy tetrahydro BaP in urine of rats, but the method was apparently not sensitive or specific enough for application to human studies. One method that has found widespread acceptance in industrial toxicology is the determination of a single marker metabolite, 1-hydroxy pyrene, by means of HPLC with fluorescence detection (Jongeneelen et al., 1987). The assay is based on the assumption that the typical PAH profile in the workplace atmosphere is roughly constant, and that the presence of metabolites of the relatively harmless pyrene could also serve as an indication of exposure to other, more carcinogenic PAHs like BaP. The sensitivity of the HPLC method did not permit the detection of BaP metabolites in workers' urine.

SCOPE OF THIS THESIS

The joint application of monitoring approaches at different levels is of crucial importance for the definition and assessment of political measures that aim at an improvement of the environment. The results from monitoring studies at the population level and analytical data on contaminant concentrations in the field can only be adequately combined when information is available on the uptake of these contaminants by organisms and on responses at the biochemical level. The techniques that will be described in this thesis offer unique possibilities to obtain this information for PAHs, even for biota in field situations. Presently available methods generally lack the selectivity and/or the sensitivity required for such studies, in particular for application in the marine environment, where concentrations of contaminants are usually low compared to most inshore locations.

In this thesis , two fluorescence-based spectroscopic techniques have been evaluated as for their usefulness for PAH and PAH metabolite analysis, i.e. Shpol'skii spectroscopy and synchronous fluorescence spectroscopy. In our laboratory considerable experience has been obtained concerning low-temperature, high-resolution (laser-based) fluorescence techniques and their application to PAH analysis (Dekkers, 1979; Cofino, 1983; Hofstraat, 1988). In the present research project the quantitative aspects of the Shpol'skii method, questioned in the literature (Lukasiewicz and Winefordner, 1972) are investigated in detail. Another important objective was to extend the applicability of Shpol'skii spectroscopy to the identification and trace level determination of relevant PAH metabolites.

With the Shpol'skii technique highly specific fluorescence spectra can be obtained for certain analytes in (poly)crystalline matrices at cryogenic temperatures. Shpol'skii spectroscopy can be used for the unambiguous identification of isomeric PAHs or for the direct determination of PAHs in complex samples. Theoretical, instrumental, and analytical aspects of the method are discussed in chapter 2.

A second technique, synchronous fluorescence spectrometry (SFS), is presented in chapter 3. SFS is less specific than Shpol'skii spectrometry, but can sometimes be very useful for rapid, low-cost screening of large numbers of samples (Vo-Dinh, 1981).

In chapter 4 the Shpol'skii technique is applied to the determination of parent PAHs in various marine environmental samples. Section 4.1 describes the qualitative Shpol'skii analysis of PAHs in HPLC fractions from a marine suspended matter sample. The goal of this study was the assessment of chromatographic peak purity and the identification of unknown compounds. The applicability of lamp excited Shpol'skii spectrometry to quantitative environmental monitoring is evaluated in sections 4.2 (synthetic mixtures) and 4.3 (reference sediment material). In both cases the results are compared with analytical data obtained with other techniques. Application of the Shpol'skii method to biological monitoring studies starts with section 4.4. Accumulated parent PAHs were determined in tissue extracts of mussel (*Mytilus edulis*) and tern (*Sterna hirundo*). The influence of high lipid concentrations on the shape, intensity and reproducibility of low-temperature spectra of crude biota extracts is studied in detail. In section 4.5 the supreme sensitivity and selectivity of laser excited Shpol'skii spectroscopy (LESS) for PAH analysis in sediment and biota samples is demonstrated.

Chapter 5 deals with the Shpol'skii spectroscopic determination of PAH metabolites in fish bile, in order to biomonitor PAH uptake from the aquatic environment. Hydroxy derivatives of benzo[a]pyrene (BaP) were determined in bile of flounder (*Platichthys flesus*) after injection with BaP or after exposure to sediments with a varying degree of pollution. Using chemical derivatization and LESS detection, an analytical protocol was developed for the quantitative trace determination of 3-hydroxy BaP. The relative contribution of various uptake routes to the total exposure was also studied.

Bile samples from fish exposed to different sediments (containing mainly PAHs of pyrolytic origin) were found to exhibit roughly constant PAH metabolite profiles, which were dominated by a single compound: 1-hydroxy pyrene. The latter could be used as a marker metabolite for PAH stress in the aquatic environment, in a similar way as has been suggested for the monitoring of occupational exposure. In section 6.1 synchronous fluorescence spectrometry (SFS) is shown to be an extremely rapid and simple method for the determination of 1-hydroxy pyrene in fish bile samples. Using the SFS method, PAH exposure levels were determined during the course of a number of fish surveys at the North Sea, and at coastal, estuarine and freshwater sites in the Netherlands (section 6.2). Possible associations with observed hepatic tumor incidences in fish populations are discussed.

In chapter 7 the analytical performance of laser excited Shpol'skii spectrometry and of HPLC with (laser induced) fluorescence detection is evaluated for the determination of 3-hydroxy BaP in urine samples of cokeoven workers and of occupationally non-exposed referents. Concentrations are compared to 1-hydroxy pyrene levels determined in the same samples, in order to investigate the usefulness of the latter as a marker metabolite for routine monitoring of PAH exposure.

Chapter 8 contains a brief summary of the most important conclusions, and some general remarks concerning environmental and biological monitoring and the importance of advanced spectroscopic methods for the success of those studies.

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CHAPTER 2

SHPOL'SKII SPECTROSCOPY

2
SHPOL'SKII SPECTROSCOPY

INTRODUCTION

Spectroscopy can be defined as the study of the interactions between matter and electromagnetic radiation. The term optical spectroscopy is usually reserved for those studies that involve visible, ultraviolet, or infrared light. Through the absorption of light, molecules (or atoms) may be promoted to a higher electronic state (S_1, S_2, etc., see Fig. 1). The energies and probabilities of these transitions can be studied by recording an (electronic) absorption spectrum. In the condensed phase, relaxation of the excited molecule to the lowest vibrational level of the S_1 state is usually very fast. The remaining excitation energy stored in the molecule may also be lost via internal conversion and vibrational relaxation, or may be transferred intermolecularly to other chromophoric groups in the sample or intramolecularly within the same molecule. For a limited number of compounds, however, the return from S_1 to the electronic ground state S_0 is accompanied by the emission of a photon (fluorescence). In some cases, phosphorescence from the lowest vibrational level of the triplet state T_1 can also be observed. The term luminescence includes both types of emission phenomena, as well as emission that results from a chemical reaction (chemiluminescence, bioluminescence). Fluorescence and phosphorescence spectra yield information on the vibrational levels of the electronic ground state S_0, and are as such complementary to the absorption spectrum. The transition between the absolute ground state and the lowest vibrational level of the S_1 state is called the 0-0 transition. This transition usually corresponds to the lowest energy band of the absorption spectrum and to the highest energy band of the fluorescence spectrum. In liquid solutions, an energy difference between the two 0-0 bands (the Stokes' shift) is often observed as the result of reorientation of the solvent shell within the lifetime of the excited state. This phenomenon can be exploited in synchronous fluorescence spectrometry, SFS (see chapter 3), but is absent in low temperature solid solutions.

Fluorescence-based techniques are widely recognized as useful tools in analytical chemistry, especially because of their high inherent sensitivity (zero-background measurement) Obviously, the fact that only a restricted group of analytes displays fluorescence implies that the method is on the one hand not universally applicable, but at the same time more selective. Polycyclic aromatic hydrocarbons (PAHs) are usually good fluorophores. Several fluorescence-based techniques have been developed to study PAHs for analytical purposes. For the determination of mixtures of PAHs, for instance in petrochemical or environmental samples, fluorimetric detection usually takes place after some form of (chromatographic) separation. Separation is necessary, as the fluorescence spectra of molecules often consist of rather broad bands that provide little information on the identity of the compound. Furthermore, fluorescence spectra of different compounds often show severe overlap. Identification and proper quantitation of analytes in a mixture is, under conventional conditions, not possible.

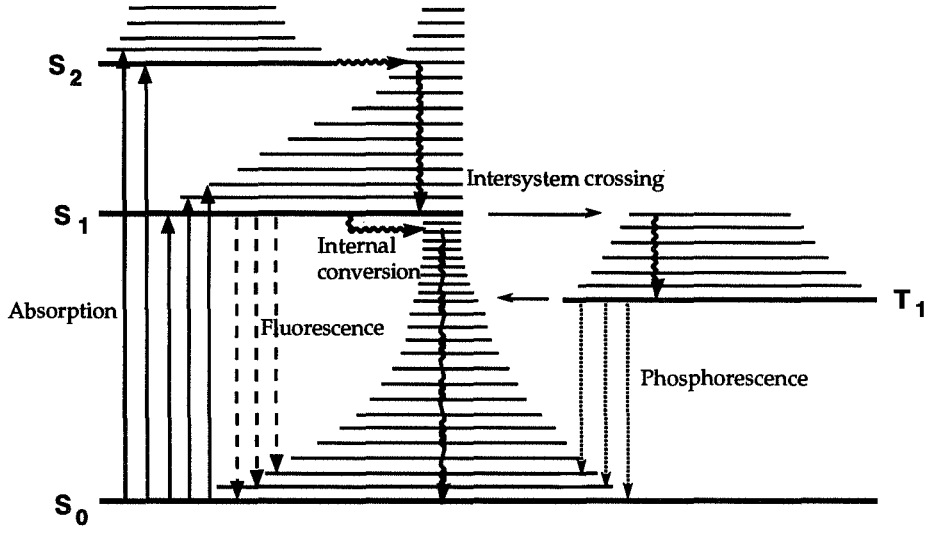

Fig. 1 Jablonski diagram of molecular system, showing relevant electronic transitions and decay processes. S = singlet state; T = triplet state.

A simple method that leads to some spectral simplification and reduced overlap is known as synchronous fluorescence spectrometry (SFS). Theoretical, instrumental, and analytical aspects of SFS are discussed in chapter 3; the practical applicability of SFS to biological monitoring of PAH exposure in the aquatic environment is demonstrated in chapter 6.

A more significant increase in spectral resolution may be obtained by means of several low-temperature techniques: fluorescence line-narrowing spectroscopy (FLN), supersonic jet spectroscopy (SJ), matrix isolation spectroscopy (MI), or Shpol'skii spectroscopy (SS). The major emphasis of this thesis will be on application of the Shpol'skii method to the analysis of PAHs and PAH metabolites (chapters 4, 5, and 7). In the present chapter, a brief overview of some of the theoretical and practical aspects of the Shpol'skii method will be provided, necessary to understand and appreciate the analytical possibilities offered by the Shpol'skii effect. Excellent review papers and book chapters have appeared on Shpol'skii spectroscopy (Nurmukhametov, 1969; de Lima, 1985; Nakhimovsky et al., 1989), or on high-resolution low-temperature spectroscopy in general (Wehry and Mamantov, 1981; Hofstraat et al., 1988).

THEORY

Spectral band broadening

As depicted in the Jablonski diagram in Fig. 1, fluorescence involves the emission of a photon with a quantum energy $E = h\nu$ that corresponds to the energy difference between the lowest vibrational level of the S_1 state and one of the vibrational levels of the electronic ground state. Fig. 1 would suggest that a fluorescence emission measurement would yield a spectrum that consists of a number of discrete lines, but that is usually not observed in molecular systems owing to several spectral broadening processes: in conventional fluorescence spectra of PAHs, band widths are typically 200-600 cm^{-1}.

In the absence of interaction with its surroundings, the uncertainty ΔE in the energy of a transition between two states depends on the respective lifetimes τ_0 and τ_1 of those states, as stated by the Heisenberg principle: $\Delta E \geq (\tau_0^{-1} + \tau_1^{-1})\ h/2\pi$, where h is Planck's constant. If one of the states involved is the ground state (of which the lifetime is practically infinite), the spectral band width of the transition will be inversely proportional to the lifetime of the excited state. The band width of a 0-0 transition of a PAH with a typical fluorescence lifetime of 10 ns would be 5×10^{-4} cm^{-1}; the band width of a vibronic transition would be some three to four orders of magnitude larger, which is still much narrower than the band widths actually observed. The Heisenberg-type of band broadening is a homogeneous broadening process, and would be observed if we could study an ensemble of isolated, motionless, identical molecules that experience no interaction with their respective surroundings.

Another type of homogeneous band broadening that needs to be considered in solid solution spectroscopy originates from electron-phonon interactions. Briefly, this type of broadening results from the fact that during an electronic transition one or several lattice vibrations of the matrix (phonons) may be simultaneously excited. As many different phonon energies may be involved, the electron-phonon line appears as a broad continuum (phonon wing) at the low-energy side of the phonon-free electronic transition (zero-phonon line, ZPL). In absorption spectroscopy the phonon wing lies at the high-energy side of the pure electronic transition. The relative intensities of the ZPL and the phonon wing are strongly dependent on the temperature and on the magnitude of the solute-matrix interactions. Fig. 2 depicts a typical ZPL-phonon wing distribution in the case of relatively small host-guest interactions at low temperature. In the case of PAHs in n-alkane matrices (the Shpol'skii technique), temperatures of 30 K and lower are usually sufficient to observe emission spectra that are largely free of phonon broadening, but substantial broadening is observed at higher temperatures, for instance when liquid nitrogen (77 K) is used as cryogenic substance. An illustration of the temperature-dependence of the Shpol'skii fluorescence emission is given in section 4.5 (Fig. 1). Very low (liquid helium) temperatures are necessary to observe intense zero phonon transitions in systems with stronger solute-matrix interactions, as in the case of phenolic analytes in water-glycerol glasses (Jankowiak and Small, 1989).

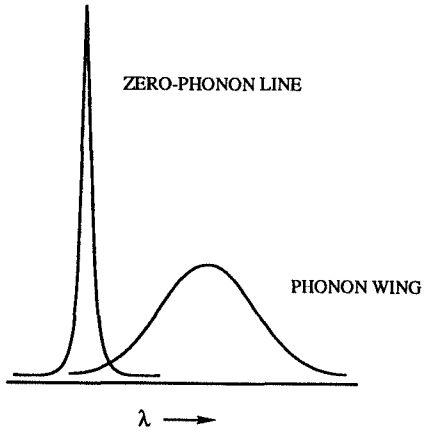

Fig. 2 Schematic representation of phonon-free line (ZPL) and phonon wing in emission spectrum in case of relatively small host-guest interaction at low temperatures.

Inhomogeneous broadening is observed when the analyte molecules in a liquid solution or in an amorphous glassy matrix are surrounded by different solvent cages. Each individual molecule will experience a different influence from its immediate surroundings on S_1-S_0 energy levels. When recording a low-temperature fluorescence spectrum of such a sample, each transition will appear as a Gaussian distribution of narrow lines, with a total band width of typically several hundred cm^{-1}. Very often matrix inhomogeneity is the major contribution to the overall band width observed in conventional fluorescence spectroscopy.

High-resolution fluorescence spectroscopic techniques

One way to observe narrow-banded emission from a compound in an inhomogeneous matrix is to use an excitation source with a band width that is much smaller than that of the inhomogeneously broadened absorption transition of the analyte (e.g. a laser), and thus make a selection among the individual analyte molecules. Only those molecules whose individual (narrow-banded) absorption spectra coincide with the energy of the laser line will be excited. When the analytes and the solvent cages do not move within the fluorescence lifetime of the analyte (as in a low-temperature solid solution), the excitation selection is maintained and a narrow-banded emission spectrum can be observed. This technique, known as site-selection spectroscopy or fluorescence line-narrowing spectroscopy, was first applied to PAHs by Personov et al. (1972). More recent papers have demonstrated the identification of PAH adducts in undigested DNA (Sanders et al., 1986; Jankowiak and Small, 1989), and the identification of PAHs and PAH derivatives on thin-layer chromatographic plates (Hofstraat et al., 1985a).

Alternatively, one could attempt to diminish or remove the inhomogeneous broadening induced by the matrix. One obvious solution would be to remove the matrix completely and study the analyte in the gas phase. High-temperature gas phase measurements suffer from other broadening effects, Doppler broadening and population of higher vibrational, rotational and translational states. However, a gas-like phase at low temperatures can be obtained if the analyte is mixed with an inert carrier gas and supersonically expanded through a small orifice into high vacuum. Supersonic jet spectroscopy is most often used to obtain high-resolution excitation spectra: a dye laser is applied to scan the absorption profile, while total fluorescence or multiphoton ionization is used as detection principle. At present, the technique seems most suitable for fundamental spectroscopic studies, but analytical applications have also been reported (Hayes and Small, 1983).

The influence of matrix inhomogeneity is also decreased when the absolute magnitude of the solute-solvent interaction is small. The technique in which analyte molecules are deposited onto a cryogenic surface, isolated from each other by inert molecules (e.g. argon, nitrogen) is called matrix isolation spectroscopy. The technique was traditionally applied to the study of very reactive species. However, the fact that the method provides transparent, solid solutions with minimal solute-matrix interaction would make the technique also very useful for analytical purposes, as was first demonstrated by Stroupe et al. (1977). Typical band widths of PAH emission lines in inert gas matrices (lamp excitation) are 50-100 cm^{-1}, which corresponds to approximately 1-2 nm. This indicates that the influence of matrix inhomogeneity has been strongly reduced, but not yet completely eliminated (Stroupe et al., 1977). Much better resolution can be obtained when the vapor deposition method is carried out with n-alkanes, that form a (poly)crystalline matrix at low temperatures. This way, matrix isolation is combined with Shpol'skii spectroscopy (Wehry and Mamantov, 1981).

Inhomogeneous broadening can also be reduced when the analyte is incorporated into a crystalline matrix, provided the analyte assumes a specific orientation in a specific site. In the ideal case, every individual molecule will experience exactly the same static interactions with the crystal lattice. Narrow-banded emission has been observed for mixed crystals, such as naphthalene in durene (McClure, 1955). When the analyte and matrix molecules have very similar geometrical and physical properties, the binary mixture will remain in thermal equilibrium during the cooling process and single crystals can be grown. For analytical applications, however, the process of growing such crystals would be very tedious, and for most analytes an appropriate matrix solvent cannot be found. The method is obviously not suited for the analysis of mixtures.

The Shpol'skii effect

Shpol'skii and coworkers (1952) observed a dramatic line-narrowing effect in the fluorescence spectrum of coronene on rapid cooling to 77 K in n-hexane or n-heptane solutions. The use of other low-temperature organic matrices did not produce high-resolution spectra. The

phenomenon, which soon became known as the Shpol'skii effect, is illustrated in Fig. 3a and b, which depicts the fluorescence spectra of benzo[k]fluoranthene in n-octane at room temperature and at 26 K, using the same experimental setup. Since the total fluorescence intensity is at least equal and often even higher at low temperatures, the line-narrowing effect results at the same time in an increase in signal height of two orders of magnitude. The sensitivity of the Shpol'skii technique will be discussed in more detail in the section on analytical applications.

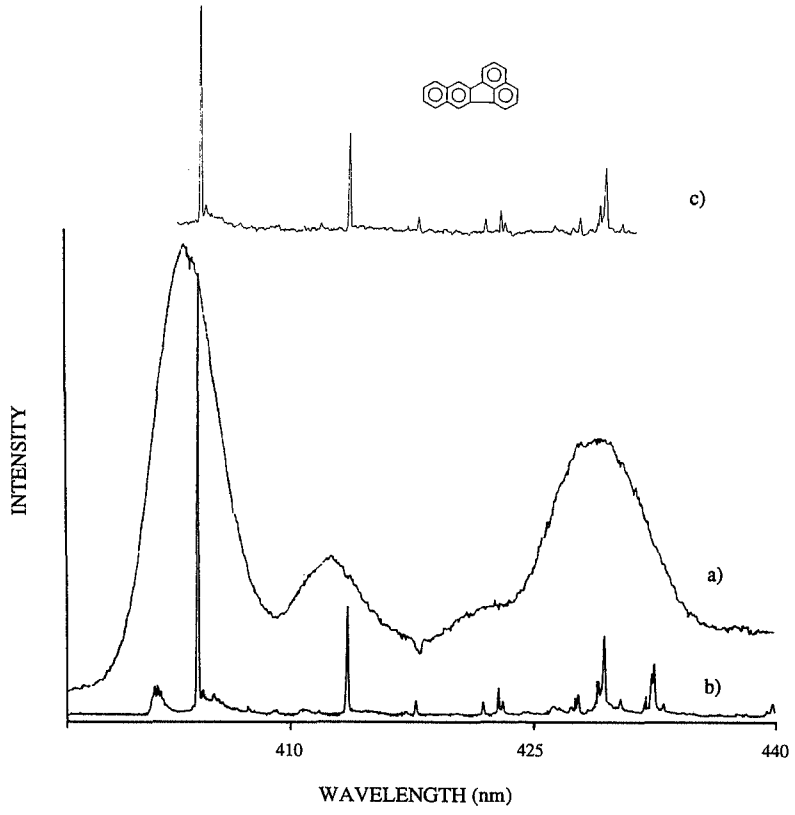

Fig. 3 Emission spectra of benzo[k]fluoranthene in n-octane. a) Room temperature spectrum, conc = 10^{-4} M. b) Shpol'skii spectrum at 26 K, conc. = 10^{-6} M. Spectra a and b were recorded using the same experimental settings; intensities are on the same scale. c) Standard reference spectrum of benzo[k] fluoranthene in n-octane; conc., 1.3 x 10^{-6} M; temperature, 15 K (from Karcher et al., 1983).

It is generally believed that the narrow-banded or quasilinear emission lines are produced by isolated molecules, trapped in the matrix during the cooling procedure and substituting one or a few solvent molecules in the (poly)crystalline lattice (Pfister, 1973a; Merle et al., 1977; Nakhimovsky, 1989). When the analyte can occupy two or more different sites in the matrix, a multiplet spectrum will be observed, as the individual spectra of different subsets of molecules are shifted with respect to each other as the result of different matrix interactions (different 0-0 energies). The Shpol'skii spectrum of 1-hydroxy benzo[a]pyrene in n-octane (Fig. 4) is an example of a doublet spectrum. The intensity ratio of the various emission lines within a multiplet is the same for each transition and reflects the distribution of the analyte molecules over the various sites. When a narrow-banded excitation source (e.g. a dye laser) is tuned to a narrow-banded absorption transition of one of the analyte subsets, a single-site spectrum will be observed, as was first demonstrated by Vo-Dinh and Wild (1973). This technique is in fact a combination of FLN and Shpol'skii spectroscopy.

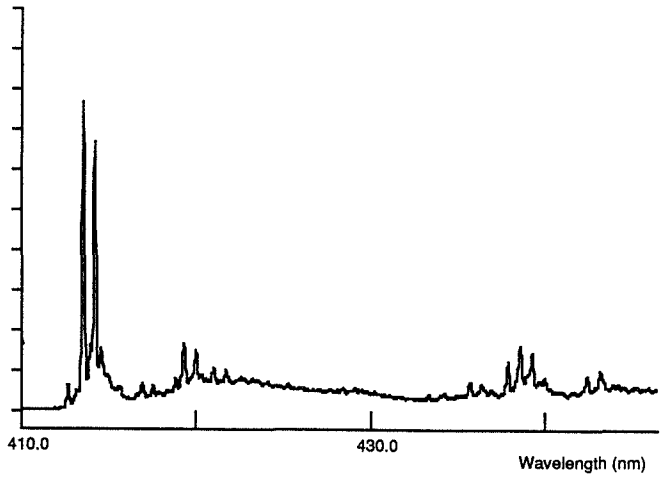

Fig. 4 Double-site spectrum of 1-hydroxy benzo[a]pyrene in n-octane at 10 K; conc., 5 x 10^{-6} M; lamp excitation 295 nm.

The spectral band widths that can be observed in Shpol'skii systems at temperatures of 20 K and lower are typically 2-10 cm^{-1} (ca. 0.1 nm). Abram and coworkers (1974) showed that the line width of a vibronic emission band of perylene in n-octane at 4.2 K could be reduced from 4 cm^{-1} to the instrumental limit of 0.4 cm^{-1} by selective excitation with a laser. This illustrates that some inhomogeneous broadening is still present in Shpol'skii systems. In practice, however, the line-narrowing induced by the matrix alone (in a lamp excited experiment) is often sufficient for isomer-specific identification and for the determination of complex mixtures (see sections 4.1 - 4.4).

The non-equilibrium nature of Shpol'skii systems

In contrast with the truly isomorphic binary systems that are used in the mixed-crystal technique, the solid solutions giving rise to the Shpol'skii effect are usually not in thermal equilibrium. Infinitely slow cooling rates would cause separation of phases and the formation of analyte aggregates instead of isolated molecules. The Shpol'skii effect is observed when the solute and solvent molecules show partial geometrical conformity and form metastable supersaturated solutions during rapid freezing (Nakhimovsky, 1989). The analyte molecules are trapped in the solid matrix as isolated molecules, but heating of the frozen sample to a point just below the melting temperature of the matrix (annealing), followed by a second cool-down often results in a change of the multi-site structure of the spectrum, or in the complete disappearance of the quasilinear emission (Pfister, 1973b), thus indicating the non-equilibrium nature of the system.

Whether a given analyte will produce Shpol'skii-like emission in a particular matrix depends on the compatibility of the physical and geometrical properties of the host-guest combination. For instance, naphthalene "fits" in an n-pentane crystal, but yields only broad-banded emission in n-hexane and n-heptane (see Fig. 5). This phenomenon is usually referred to as the "key and hole principle", but it appears that the geometric requirements are less stringent for most larger PAHs containing four or more rings, as they produce good Shpol'skii spectra in a range of n-alkane matrices (Dekkers et al., 1977; Pfister, 1973a).

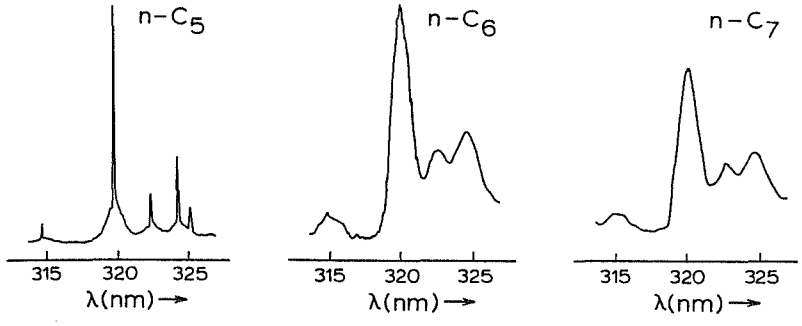

Fig. 5 Illustration of the "key and hole" rule for naphthalene in various matrices; temperature, 20 K.

For some host-guest combinations, the Shpol'skii spectrum may show both quasilinear and broad-banded emission. In that case, the appearance of the spectrum (relative intensities and site distribution), often depends critically on various experimental parameters, such as concentration, cooling regime and sample holder design. These solutes, that are not fully compatible with the matrix, are usually referred to as "type β" compounds, according to the nomenclature of Rima et al., (1984). Especially the cooling rate is often considered as a critical parameter; the cooling process consists of three phases (cooling of the liquid, solidification of the matrix, and further cooling of the solid solution), each of which may have a different influence on the metastable system formed (Nakhimovsky et al., 1989). Hofstraat and coworkers (1989) studied the emission of the non-planar PAH acenaphthene in frozen n-hexane at different concentrations, using different sample holders and cooling methods. The authors concluded that the broad-banded emission originated from analyte molecules that had been frozen out of the crystal lattice at the point of matrix solidification, and had concentrated in interstitial amorphous regions. Such poorly compatible analytes are most efficiently trapped in crystalline sites when the cooling regime and the sample holder design are optimized for instantaneous solidification of the sample. An illustration of the sensitivity of the spectra of "type β" compounds to experimental conditions is given in Fig. 6, which presents the Shpol'skii emission spectra of 3-hydroxy benzo[a]pyrene measured in two different laboratories. It was stated by Lukasiewicz and Winefordner (1972) that the irreproducible nature of the spectra of this type of compounds would hamper the application of the Shpol'skii technique to quantitative analysis.

Fortunately, there are also many compounds, including most larger PAHs that are listed as priority pollutants by the USA Environmental Protection Agency, that show very good compatibility with one or several n-alkane matrices. Over a wide range of concentrations, these "type α" compounds (Rima et al., 1984) yield reproducible, high-quality Shpol'skii spectra, which are more or less independent of the cooling rate. Comparison of the Shpol'skii emission spectra of benzo[k]fluoranthene in Figs. 3b and c, also measured in different laboratories, illustrates this point. For this group of compounds the Shpol'skii method is very suitable for both qualitative and quantitative analysis, as will be demonstrated in chapters 4 and 5. Alternatively, one could also attempt to transform less compatible analytes into "type α" compounds by means of a chemical reaction (Weeks et al., 1990), as will be demonstrated for mono-hydroxy benzo[a]pyrene derivatives in chapter 5.

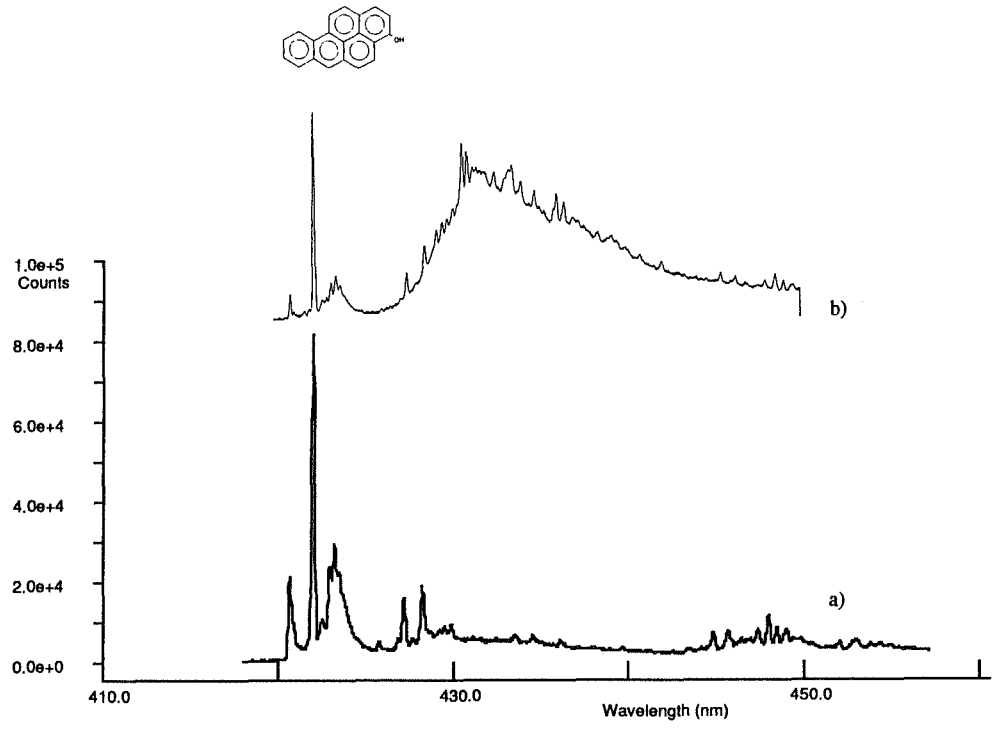

Fig. 6 Shpol'skii spectra of 3-hydroxy benzo[a]pyrene in n-octane. a) Conc. = 1.4 x 10^{-5} M; lamp excitation 300 nm; temperature, 10 K; (from Ariese et al., 1993a). b) Standard reference spectrum; conc., 5 x 10^{-6} M; lamp excitation 307 nm; temperature, 15 K (from Karcher et al., 1991).

INSTRUMENTAL ASPECTS

Schematically, an instrumental setup for Shpol'skii fluorescence measurements consists of the following modular components: 1) Excitation source, often with some type of wavelength selector 2) Low temperature sample holder. 3) High-resolution emission monochromator. 4) Detector. At present, no complete turn-key instruments are available on the market, but a Shpol'skii setup can be assembled from commercially available components. The various options for each of these modules, with their specific advantages and disadvantages for Shpol'skii measurements, will be briefly considered in this section. For a more detailed discussion on the use of light sources, monochromators, and detectors in analytical spectroscopy, the reader is referred to Ingle and Crouch (1988).

Excitation sources, lamps

The Shpol'skii effect is a matrix-induced phenomenon; the use of narrow-banded (laser) excitation can be advantageous, but is not a prerequisite for observing quasilinear spectra. Two types of lamps have been commonly used in conventional Shpol'skii measurements. Most early Soviet papers (reviewed by de Lima, 1985) report the use of mercury-vapor lamps. Mercury lamps with very high powers are available; a 1000-W Hg lamp was employed by Paturel et al. (1983). Maximum excitation power is achieved when a large fraction of the lamp output is selected. In that case, low-pass filters or broad banded interference filters are used to select a wide excitation range. Increased selectivity is obtained when the lamp is used in combination with an excitation monochromator. This does not necessarily imply a decrease in sensitivity, as the detection limit is often limited by excitation scatter. Typical excitation band widths are 2-10 nm. High-resolution excitation is employed for recording excitation spectra or for site-selective excitation. Xenon arc lamps, which produce a continuum spectrum rather than a line spectrum in the short wavelength region (Parker, 1968), have also been very popular, especially for use in combination with an excitation monochromator (de Lima 1985).

Excitation scatter from the frozen sample, the sample cell, and from the windows of the cryostat can be a major limiting factor in Shpol'skii trace analysis. Scattering from a line source can interfere with the narrow emission spectrum. Scattering from a continuum source can contribute significantly to the background noise. Lamp excitation of PAHs is usually performed at relatively short wavelengths (excitation into the S_2 or higher states) for optimum sensitivity and minimum scatter. Extra filter combinations can be used for further decrease of scatter. An interesting solution for the light scattering problem was presented by D'Silva and coworkers (1976), who applied non-selective X-ray excitation from a tungsten target and obtained typical Shpol'skii emission spectra.

For the lamp excited Shpol'skii experiments described in chapter 4 of this thesis, a 450 W xenon lamp was employed in combination with an excitation monochromator. A chemical filter made of 25 % aqueous nickel sulfate solution was used as an extra bandpass filter.

Laser excitation

It was evident from the early studies by Shpol'skii and Girdzhiyauskaite (1958) that for PAHs under Shpol'skii conditions also the S_1-S_0 part of the absorption spectrum consists of narrow lines. The line width of transitions to higher states is usually much broader, as a consequence of the Heisenberg principle (the lifetimes of those states are extremely short). Information on the absorption transitions is most conveniently derived from a high-resolution excitation scan. More recently, a double-beam instrument was developed for the measurement of absorption spectra of fluorescent compounds in frozen solutions (Soulignac and Lamotte, 1987). From the narrow-banded absorption spectra in the spectral atlas of Nakhimovsky et al. (1989), it is clear that an impressive increase in selectivity can be obtained when the energy of the narrow-banded light source (e.g., a dye laser) overlaps with a specific narrow transition of the analyte.

Vo-Dinh and Wild (1973) used an argon ion laser to study the site distribution of coronene in n-heptane. One of the UV lines of the argon ion laser happened to coincide with one of the lines of an absorption doublet. Evidently, the specific advantages of selective excitation are only fully exploited if the laser system is tunable within the most specific wavelength region of the low temperature absorption spectrum of the particular analyte under investigation. With a nitrogen laser-dye laser combination, Yang and coworkers (1981a) were able to selectively excite and detect alkylated benz[a]anthracenes in a complex mixture of isomers.

Nowadays, several types of lasers and dye lasers are available that -in combination with frequency doubling, frequency tripling, frequency sum mixing or Raman shifting techniques- cover the complete range of relevant wavelengths. It is important to make a distinction between pulsed or continuous wave (CW) lasers. CW lasers that can be used for PAH excitation include the helium cadmium (He-Cd) laser (335 and 442 nm), and the argon ion laser (several lines between 334 and 529 nm). The He-Cd laser was first tested by Causey and coworkers (1976), who observed that non-selective laser excitation with a 3 mW low-power laser resulted in little or no improvement in PAH detectability, compared to excitation with a 125-W medium-pressure Hg-lamp. The output of the He-Cd laser is too low for pumping a dye laser. Large-frame argon ion lasers usually provide sufficient power to pump a CW dye laser. Wavelengths from 400 nm onwards are available when the argon ion laser is operated in the UV mode (see also chapter 7). If shorter excitation wavelengths are required, a mode-locked argon ion laser can be used to synchronously pump a rhodamine dye, the output of which may subsequently be frequency doubled (van de Nesse et al., 1993).

Among the pulsed lasers, different types of excimer lasers (e.g., XeCl, output 308 nm; XeF, output 351 nm) or a nitrogen laser (337 nm) are suitable for direct pumping of a dye laser that covers the desired wavelength range. The pulsed (flashlamp operated) Nd:YAG laser with a fundamental output of 1064 nm can be frequency doubled or frequency tripled for pumping a specific dye laser. Nd:YAG lasers that operate in the CW mode are also available, but are less suitable for pumping purposes. Obviously, the type of laser excitation mode, CW or pulsed, will determine what type of detector and detection mode will be most appropriate (see below).

An important advantage of all laser systems is the spatial coherence; beam divergence can be less than a mrad, which means that the beam is easily focussed on a small sample volume. In our department frequent use is made of quartz optical fibers to transfer laser light from neighboring laboratories to the sample compartment.

As a disadvantage, it should be mentioned that the tuning range of laser dyes is often rather limited (typically 20-40 nm). Frequent change of the dye solutions can be a tedious and costly procedure, unless several dye pumps and easily exchangeable dye cuvettes are available. For these reasons, the laser appears less suited for the selective excitation of a range of analytes with very different 0-0 energies. However, for the laser excited Shpol'skii measurements of benzo[a]pyrene metabolites presented in chapter 5, the Nd:YAG setup could be easily switched from frequency mixing (for selective excitation around 420 nm) to frequency doubling of the dye

laser output (for non-selective excitation around 350 nm). A promising new development is the use of solid-state tunable lasers, such as titan-sapphire lasers, which are tunable over several hundreds of nm. When pumped in the pulsed mode, the output of these lasers can be frequency doubled to yield a broad tuning range that could be used for selective excitation of a wide range of compounds.

Cryostats and sample holders

The Shpol'skii effect was first observed at a temperature of 77 K, the boiling point of liquid nitrogen. In most experiments carried out at that temperature, the sample was contained in an ordinary glass or quartz test tube and simply immersed in a Dewar flask with liquid nitrogen. In order to change the freezing conditions, the sample cell can be precooled at a slower rate by cold nitrogen vapor prior to immersion (Nakhimovsky et al., 1989). Causey and coworkers (1976) presented an alternative sample holder design, in which a quartz cell in a copper holder was mounted to the outside of the liquid nitrogen container, and cooled by thermal conductance. Spectral resolution is improved when the temperature of the nitrogen cryostat is lowered from 77 K to 63 K. This was achieved by pressure reduction to 10^{-2} torr, thus producing solid nitrogen (Colmsjö and Stenberg, 1977)

Several types of helium cryostats are available to reach even lower temperatures. The helium bath cryostat is a well-isolated Dewar flask in which the sample can be immersed (usually after precooling in liquid nitrogen). The helium container is surrounded by vacuum and liquid nitrogen mantles for thermal insulation. Excitation and emission collection usually takes place through a set of windows, which may introduce considerable extra scatter, but can also be achieved from the top by means of quartz light guides (Isaacs and Heiman, 1987; F. Ariese, unpublished results). The normal boiling temperature of liquid helium is 4.2 K, but this can be reduced by pressure reduction (obviously involving extra costs). Continuous-flow helium cryostats can also be used: in these devices, the sample is cooled by thermal conductance, as liquid helium is flushed through a heat exchanger connected to the sample holder. The minimum temperatures that can be obtained are typically 4-10 K. The advantage over the bath cryostat is a reduction of scattered light (no boiling helium and fewer windows in the light path). Closed-cycle helium refrigerators have become increasingly popular for Shpol'skii measurements (Garrigues et al., 1981). In these cryostats helium, the circulating medium, is expanded in the cold station and cools the sample through thermal conductance. Depending on the number of cold stages of the instrument, temperatures of typically 10 or 20 K can be reached. Garrigues et al. (1981), comparing the spectral resolution of PAHs at 15 and 4.2 K, concluded that for PAH analysis in n-alkane matrices (relatively small electron-phonon coupling) the ease and low cost of operation of the closed-cycle refrigerator (no consumption of helium) outweighs the advantage of slightly better resolution obtainable with the bath cryostat.

With most continuous-flow and closed-cycle helium cryostats, it may take 30-60 minutes to reach the final temperature. The process can be accelerated by immersing the sample holder (attached to the cold station of the cryostat) in liquid nitrogen, followed by slower cryocooling of

the frozen sample to the final temperature. Especially when slow cooling procedures are applied, it is advantageous to construct a sample holder in which several samples can be frozen simultaneously (Garrigues et al., 1981; Paturel et al., 1983; Elsaiid et al., 1988). In order to be able to study the sample at other temperatures than at the minimum temperature, and to accelerate the warming-up of the sample at the end of the experiment, the cold station can be connected to an electrical heater.

The cooling procedure obviously affects the freezing rate of the sample, and may thus have an influence on the shape of the spectrum for certain analytes. Which method results in the most efficient trapping of isolated molecules in crystalline sites will also depend on the sample holder design. Hofstraat et al. (1989) demonstrated that the use of window materials and spacers of poor thermal conductivity (quartz and teflon, resp.) led to a slow solidification rate and to predominantly broadbanded emission in the "type β" system acenaphthene in n-hexane. Better quasilinear spectra were obtained when materials with a high thermal conductivity were used (sapphire windows, indium spacers). With the "high-conductivity" sample holder, slow cooling with the closed-cycle refrigerator yielded better spectra than fast cooling by immersion in liquid nitrogen.

The experiments described in this thesis were carried out using a recently developed sample holder, made of gold-plated copper (Fig. 7). Four 10 µl samples can be put into small depressions, and covered with sapphire windows. The sample holes have a diameter of 5 mm; the sample depth is defined by the thickness of the teflon packing (ca. 0.4 mm). The sample holder is fitted to the cold station by means of two screws; indium sheet is used for extra thermal conductivity. Visual inspection of the samples during cryocooling revealed that solidification of the matrix was very fast (<<1 s).

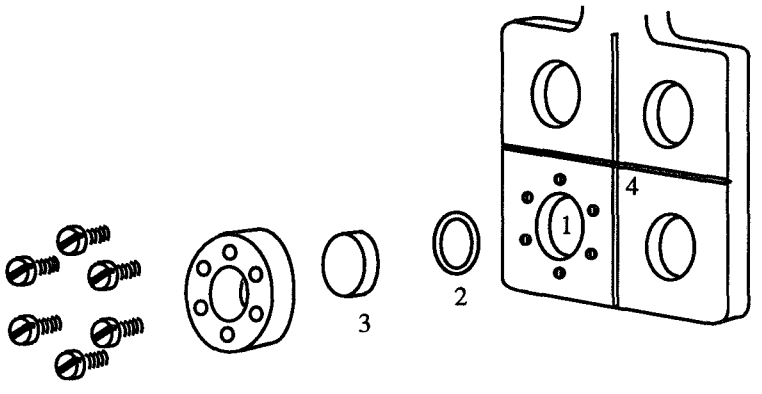

Fig. 7 Exploded view of laboratory-made sample holder 1) Sample hole 2) Disposable teflon packing 3) Sapphire window 4) Groove to retain spilled sample liquid. Sample holder and retaining rings are of gold-plated copper. Sample volume ca. 10 µl.

Emission monochromators

A monochromator of moderate or high resolution is required to observe the quasilinear emission spectrum. The spectral resolution of the instrument should preferably be less than 0.2 nm. For trace analysis, the instrument should also have a high light throughput. Holographic gratings, optimized for the wavelength area of interest, are preferred. Often the F/n number of the emission monochromator will be fairly high, which means that some care should be taken on proper focussing of the fluorescence light on the entrance slit.

If short-wavelength excitation is applied, scattered excitation light is easily rejected with an appropriate cut-off filter. If, on the other hand, the excitation wavelength is close to the emission lines of interest, as in the case of laser excitation in the S_1-S_0 absorption region, the cut-off functions of such filters are usually not sufficiently steep. In that case, the use of a double- or even triple monochromator will be very advantageous. For the laser excited Shpol'skii spectroscopy (LESS) measurements in this thesis, a Spex 1877 triple monochromator was used, in which the first two monochromators (the "filter stage") could be by-passed by switching two mirrors. Thus, the performance of the triple monochromator could be directly compared to that of a single monochromator. Figure 8 shows that the addition of the two extra monochromators resulted in a remarkable reduction in scatter: the background is reduced by two orders of magnitude, while the signal intensity was lowered by a factor of only 1.5.

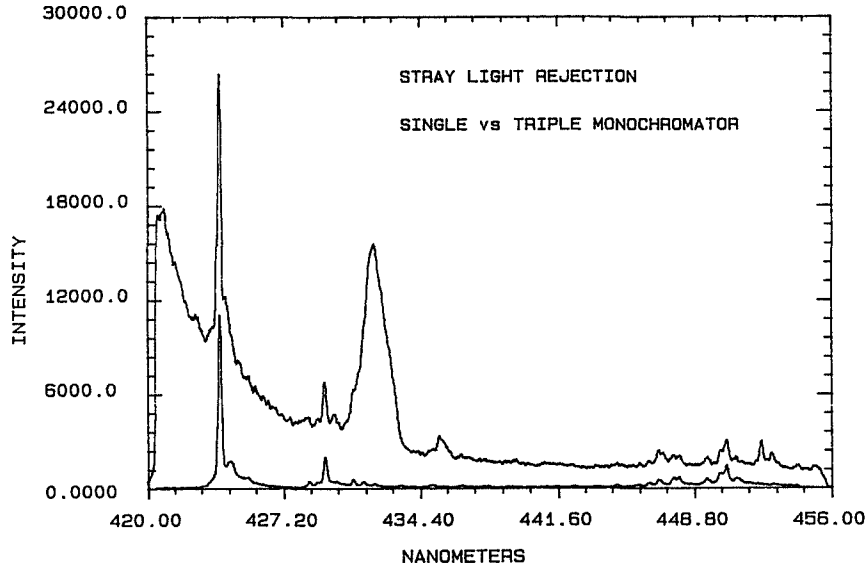

Fig. 8 Comparison of triple monochromator (bottom) with single monochromator (top). LESS spectra of 3-methoxy benzo[a]pyrene in n-octane. Excitation wavelength 418.36 nm; temperature, 26 K; no time resolution. The broad signals at 432 and 435 nm are caused by multiple reflections of the laser line within the analytical monochromator.

Detectors

During the first experiments on Shpol'skii systems, photographic plates, fitted to the exit port of a spectrograph, were in general use for detection. Later, photomultiplier tubes (PMTs), in combination with a scanning monochromator, gained in popularity. The optimum PMT choice depends on the wavelength sensitivity and gain required. Detector dark current is substantially reduced by cooling the PMT to -20 °C or lower (for instance using thermoelectrical Peltier devices), and by applying photon counting techniques. For time-resolved fluorescence measurements the response time of the PMT should be very short. For such measurements the PMT can be coupled to a boxcar integrator that samples the PMT signal only after a specific delay time and during a specific gate width. Alternatively, a pulser circuit can be used that deactivates the PMT during the laser pulse. The latter approach is especially suitable for the measurement of long-living luminescence (F. Ariese, unpublished results).

More recently, multichannel detectors have become available that are particularly useful in high-resolution spectroscopy. These detectors are mounted in the (exit) focal plane of the monochromator after removal of the exit slit. The monochromator is thus turned into a spectrograph; measurements are usually carried out at a fixed wavelength position, although operating the detector in the scanning mode can have certain advantages (Knoll et al., 1990). The intensified linear diode array (ILDA) detector consists of typically 512 or 1024 separate photodiodes (center-to center distance ca. 25 μm), that release electrons on the absorption of light. At the end of an integration cycle defined by the operator, the accumulated charge in each photodiode-capacitor pair is sampled, digitized, and fed into a computer. Since the read-out of such detectors is accompanied by considerable electronic noise, the use of an intensifier (a combination of a photocathode, a multichannel plate, and phosphor screen that multiplies the number of incident photons with a gain of typically 10^3) is indispensable for the detection of low light levels. Fast gating of the ILDA detector is possible by means of a fast pulser unit that switches the photocathode on and off at specific times.

Another type of multichannel detector is the charge-coupled device (CCD) detector. These detector chips consist of a plane of semiconductor material and a fine electronic network that divides the chip into a matrix of small squares (pixels). Typical CCD chips presently contain 512 x 512 pixels of approximately 20 x 20 μm each. As the read-out noise of CCD chips is much lower than that of photodiodes, CCD detectors do not necessarily require an intensifier. With CCD detectors two-dimensional spectral information can be recorded (Christian et al., 1981), but in case of ordinary one-dimensional spectroscopic measurements the charge accumulated in each column can be summed ("binned") for extra sensitivity. A scintillator dye can be applied to the CCD chip for extension into the UV region.

In our experience, ILDA and CCD detectors offer roughly comparable sensitivities when applied to Shpol'skii analysis. The ILDA detector seems more appropriate when fast gating is required. The resolution of these multichannel detectors is of course defined by the width of the photodiodes/pixels and by the linear dispersion of the spectrograph applied. In practice, the

actually observed resolution corresponds to 2-3 resolution elements, as the result of cross-talk or loss of spatial resolution in the intensifier unit. Depending on the required spectral resolution, a spectrograph with an appropriate linear dispersion should be chosen accordingly. For Shpol'skii measurements a linear dispersion of typically 5-20 Å/mm could be used; this means 2-8 resolution elements per 0.1 nm, the typical linewidth of PAH spectra in n-alkane matrices.

We used the Jobin-Yvon HR1000 monochromator with two separate exit slits for a direct comparison of an ILDA detector (Princeton Instruments IRY 1024 GRB) with a Peltier-cooled PMT (Philips XP 2020Q, operated at 2500 V, coupled to a Stanford Research SR400 photon counter). Shpol'skii spectra of benzo[a]pyrene in n-octane (lamp excitation, 297 nm; temperature, 26 K) were recorded using slit widths of 100 μm, corresponding to a spectral resolution of 0.08 nm. In the PMT measurement, a spectral range of 20 nm was scanned in 1000 s (scan speed, 0.02 nm/s; integration time, 1 s). The range covered by the ILDA detector was also 20 nm; a detector exposure time of 10 s yielded a spectrum with a roughly similar signal-to-noise ratio as in the PMT scan. In conclusion, the sensitivity of the ILDA per resolution element was about an order of magnitude lower than the PMT, but nevertheless an overall decrease in analysis time of two orders of magnitude was realized.

A probably even more important advantage of the multichannel detector is that each data point of the complete spectrum is equally affected by light source instability (slow drift, flicker noise) or by photochemical decomposition of the analyte. These factors will be particularly important when lasers are used for excitation (see also chapter 5).

ANALYTICAL APPLICATIONS

Types of compounds studied

The Shpol'skii effect has now been observed for a rather wide range of compounds, which usually share at least two of the following molecular properties: 1) planar rather than bulky conformation, 2) rigidity, 3) restricted polarity. These requirements are a logical consequence of the nature of the Shpol'skii effect: individual analyte molecules must assume one (or only very few) well-defined orientations within specific sites in the (poly)crystalline matrix. Low polarity is required as the host-guest interactions should be small, and because the analyte should form a supersaturated solution during cooling of the sample. For these reasons, most compounds that have shown the Shpol'skii effect so far contain an aromatic moiety and show only a limited degree of conformational freedom, although there are a few exceptions.

Nurmukhametov (1969) reviewed the early Russian literature; most applications concerned fundamental spectroscopic studies. Narrow-banded fluorescence had been obtained for: PAHs, aromatic radicals, alkylated PAHs, aromatic hydrocarbons containing an N-heterocycle, such as quinoline, polyphenyls and diphenyl polyenes, arylethylenes, arylacetylenes, benzoxazoles, aromatic amines with restricted conformational freedom, porphyrins, and phthalocyanins. Most of the quasilinear spectra of these compounds were obtained in n-alkane matrices. Analytical

applications of the method included the determination of (carcinogenic) PAHs in such diverse samples as atmospheric air, tobacco smoke, smoked food products, bitumen and other petroleum-derived products, and in rock samples of geochemical interest (Nurmukhametov, 1969). More recently, Garrigues and coworkers (1985, 1987) studied the Shpol'skii spectra of alkylated PAHs in detail. Isomer-specific identification is important in geochemistry, in the case of environmental analysis of PAHs of petrogenic origin, and because different substitutional isomers can show very different carcinogenic properties (Hecht et al., 1976). The effect of substituents on the quasiline spectra and resolution was studied for a number of pyrene-derivatives by Colmsjö et al. (1982). A variety of polycyclic aromatic compounds containing oxygen-, sulfur, or nitrogen substituents was studied by Elsaiid and coworkers (1988), who applied site-selective laser excitation. So far, very few studies have been conducted to study PAH metabolites in low-temperature Shpol'skii matrices. Nurmukhametov (1969) noted that "Attempts to obtain an effectively resolved structure for the electronic bands of aromatic alcohols ... in n-hydrocarbon solution did not give positive results". In a later paper by Khesina et al. (1975), it was shown that some phenolic derivatives of benzo[a]pyrene (BaP) yield quasilinear spectra in n-octane matrices. The results were confirmed by Ariese et al. (1993a), who identified several phenolic isomers of BaP in bile of flounder (*Platichthys flesus*). In principle, the applicability of the Shpol'skii method to (poly)hydroxylated metabolites can be improved by transforming the hydroxy groups into less polar methoxy groups (Weeks et al., 1990). This approach will be discussed in more detail in chapter 5 (Ariese et al., 1993b).

Types of samples studied; selectivity

Here, we will give a brief overview of the diverse samples that were studied for analytical purposes. It is not our intention to provide a complete listing of all analytical applications that appeared in the literature (see de Lima, 1985; Nurmukhametov, 1969, for reviews), but rather to give an impression of the analytical utility of the method. Most reports describe the identification and/or determination of PAHs and PAH derivatives. The effort put into analysis of that class of compounds is obviously inspired by concerns about the carcinogenic activity of many members of the PAH family (data on biological activity of PAHs and PAH derivatives were compiled by Karcher et al., 1983; 1988; 1991). The same compounds can also be determined by more conventional analytical methods, such as liquid or gas chromatography. The advantages of the Shpol'skii method include the excellent sensitivity for many PAHs (Morel et al., 1991), the fact that usually no or only a limited sample clean-up is required, and the supreme specificity of the method for isomer identification (Garrigues and Ewald, 1983; Wise et al., 1988). Analytical figures of merit will be discussed in more detail below.

Non-selectively excited Shpol'skii spectrometry is often sufficiently specific for samples that contain mainly PAHs of pyrogenic origin and relatively few alkylated isomers. Hofstraat and coworkers (1985b) demonstrated the lamp excited Shpol'skii determination of a number of PAHs in a crude harbor sediment extract. Non-selective X-ray excitation was employed for the determination of PAHs in dust samples from a local power plant, but the spectra were too

complicated for proper quantitation when the same technique was used for PAH analysis in petrochemical products (Woo et al., 1980). Bark and Forcé (1990) tested the use of non-selective nitrogen laser excitation for the detection of PAHs desorbed from particulate matter. Gas phase PAHs were mixed with an n-hexane carrier gas and deposited on a cold finger held at 77 K; time-resolved detection was used for improved selectivity. Saber et al. (1991) applied the Shpol'skii technique to the determination of PAHs (mainly originating from automobile exhaust) in sediment samples from an Alpine lake. The same research group (Morel et al., 1991) compared a number of analytical techniques (SFS, HPLC, GC with various detectors, and Shpol'skii spectrometry) for the trace determination of PAHs in sediments and biota (clam, tongue sole, and shrimp) from various locations in Kuwait bay (prior to the Gulf War). The authors concluded that the Shpol'skii method was particularly sensitive and accurate for the direct determination of heavier PAHs, but that for the analysis of small, alkylated isomers (e.g., in an oil-spill situation) it should be backed up by a chromatographic technique.

For more complex analytical problems, chromatographic fractionation prior to Shpol'skii detection has been applied by several authors. Wise et al. (1988) employed the Shpol'skii technique for the identification of a number of isomeric high-molecular weight PAHs (MW = 302) in a coal tar extract after normal-phase HPLC separation. Colin and coworkers (1981) applied the Shpol'skii method to the determination of PAHs at sub-ppb levels in medicinal white oil, also after HPLC fractionation. PAHs were identified in automobile exhaust particulate matter after thin-layer chromatographic separation, vacuum sublimation of the analytes from excised spots onto a cold finger, and dissolution in the appropriate Shpol'skii solvent (Colmsjö and Stenberg, 1979). Garrigues and coworkers (1985) developed an HPLC fractionation/Shpol'skii detection procedure for the quantitation of alkylated PAH isomers in petroleum. Accurate determination of relative PAH isomer ratios can be used in geochemistry as a maturity index.

Very complex samples can often be analyzed without prior separation if selective laser excitation is used. Quantitative determination of a number of PAHs in various fossil fuel products (coal-derived fuel oil, diesel fuel marine produce, crude oil, shale oil and solvent-refined coal) was carried out by Yang et al. (1981a, b). Renkes and coworkers (1983) quantitated PAHs in particulate matter reference materials, following high-temperature extraction. Selective excitation also allowed the direct determination of heterocyclic compounds (dibenzothiophene, dibenzofuran) in crude oil (Elsaiid et al., 1988). The application of selective laser excitation for the determination of benzo[a]pyrene metabolites in fish bile and in human urine will be demonstrated in chapters 5 and 7, respectively.

Sensitivity

The relative intensities of the room temperature and 26 K spectra of benzo[k]fluoranthene (Figs. 3a and b) would suggest that the sensitivity of the Shpol'skii method is roughly two orders of magnitude better than that of conventional fluorimetry. This is of course somewhat misleading, since normally, for the measurement of a broad-banded spectrum of a liquid

solution, wider slit widths and a different sample compartment would have been chosen. It is probably more correct to state that, compared to conventional fluorimetry, the lower light collection efficiency of a high-resolution setup is compensated for by the line-sharpening effect induced by the low temperature polycrystalline matrix. For pure compounds, the sensitivities of both methods are roughly comparable; the major advantage of the Shpol'skii technique is the increase in selectivity (fingerprint identification and strongly reduced spectral overlap in mixtures). Table I lists reported sensitivities for benzo[a]pyrene in n-octane at different temperatures and employing different excitation modes. Instrumental breakthroughs have clearly resulted in a substantial improvement in sensitivity.

Table I Detection limits reported for benzo[a]pyrene.

Light source	Exc. wavelength selector	Temp.	Detection limit [a]	Reference
Xe lamp 150 W	Interference filter	77 K	0.3	Causey et al., 1976
Hg lamp 125W	Interference filter	77 K	0.15	"
He-Cd laser 3 mW	-	77 K	0.15	
Xe lamp 450 W	Monochromator	26 K	0.075	Ariese et al., ch. 4.2
Hg lamp 1000 W	Interference filter	10 K	0.025	Paturel et al., 1983
Hg lamp 1000 W	Monochromator	10 K	0.004	Saber et al., 1991
Nd:YAG/dye laser 6 mW	-	26 K	0.0012	Ariese et al., ch. 4.5

[a] Detection limits (in ng/ml) have been normalized to correspond to a signal-to-noise ratio of 3.

Quantitative analysis

As pointed out in the theoretical section, Shpol'skii systems are not in thermodynamic equilibrium and the type of metastable state that will be formed during cool-down depends on a number of experimental variables. For these reasons, the shape and intensity of Shpol'skii spectra were found to be rather irreproducible for particular compounds, and this phenomenon was believed to obstruct the application of the Shpol'skii method to quantitative analysis (Lukasiewicz and Winefordner, 1972). Later, however, numerous papers have appeared reporting accurate and precise results for compounds that are more compatible with the low temperature matrix (Yang et al., 1981a and b; Rima et al., 1982). As will be demonstrated in section 4.3, good quantitative results can also be obtained for crude sediment extracts. This would suggest that the difficulties envisaged by Lukasiewicz and Winefordner (1972) are in practice not a major barrier, provided that experimental conditions are well under control (Rima et al., 1990).

As in ordinary fluorimetry, the intensity of a particular emission line depends on many experimental factors, such as light source intensity, excitation optics, alignment of the sample cell, collection optics and detector sensitivity. Furthermore, matrix absorption, the inner filter effect, self-absorption, long-range energy transfer, and matrix homogeneity will influence the spectrum. Photodecomposition may play an important role in laser-excited experiments. For these reasons, the addition of an internal standard is often required for quantitative analysis. When the internal standard is structurally and spectroscopically very similar, most of these experimental factors are adequately corrected for. Perdeuterated PAHs are particularly useful in this respect (Yang et al., 1981b). These compounds are expected to respond to experimental conditions in very much the same way as their non-deuterated analogues, they are absent in natural samples, and do not interfere with the measurement, as their Shpol'skii spectra are shifted over typically a full nanometer, which is significant in view of the typical band widths of 0.1 nm. For the analysis of mixtures, addition of a single internal standard would be sufficient; calibration graphs can be constructed for the main emission line of each analyte against the quasilinear intensity of a known concentration of internal standard. However, if there are reasons to suspect that some experimental factor may have a different influence on the quasilinear emission intensity of the analyte and of the internal standard (different matrix effects, different photolability), then a standard addition approach should be followed, preferably in combination with an internal standard (Rima et al., 1982; see also section 4.3). Variations in light source intensity can also be corrected for by ratioing the analyte emission with the intensity of excitation scatter (Wittenberg et al., 1984).

Finally, it should be stressed that the reproducibility of the excitation wavelength must be within the absorption band width of the analyte. This is particularly important if selective laser excitation is used, and deserves special attention from the experimentator, since it is not corrected for by an internal standard (chapter 5; Ariese et al., 1993b).

CONCLUSIONS

Shpolskii spectrofluorimetry can be a very useful technique for the identification and/or determination of PAHs in complex samples. The quasilinear spectra can serve as fingerprints when unambiguous identification is important. Especially distinction between isomers (equal masses and very similar structures) is important, as this is often not possible with the chromatographic techniques that are routinely applied to PAH analysis.

The choice of equipment depends largely on the type and complexity of the samples to be studied. Lamp excitation is applicable to a wide range of different compounds, while lamp instability is often negligible. Laser excitation into a narrow S_1-S_0 absorption transition is more specific and sensitive, and is usually required for more complex analytical problems. However, photochemical decomposition, instability, and the limited tuning range of many pump laser/dye laser combinations can be important practical limitations. For Shpol'skii measurements of PAHs in n-alkane matrices the closed-cycle helium cryostat appears most suitable. For specific host-

guest combinations the cooling procedure and sample holder design deserves extra attention. A good emission monochromator, with a spectral resolution better than 0.2 nm, is required. Double or triple monochromators will be especially useful if selective laser excitation close to the emission wavelengths is used. Multichannel detectors can offer a substantial reduction in measurement time compared to photomultiplier tubes, which facilitates optimization of the experimental setup and diminishes the risks of photochemical decomposition. Within the range of the multichannel detector the complete spectrum is equally affected by light source instability.

The Shpol'skii method can also be used for quantitative purposes, especially for "type α" compounds that yield reproducible, good quality spectra. Optimization of the cooling procedure leads to better reproducibility. Improved excitation and detection technologies allow the analysis of more dilute samples,which diminishes factors like matrix absorption, matrix distortion, self-absorption or aggregation. Perdeuterated compounds are very suitable as internal standards; for some applications a standard addition procedure, in combination with an internal standard, must be applied.

In this thesis, the identification power of Shpol'skii spectrometry will be demonstrated in section 4.1. The isomer-specificity of the technique is illustrated for mono-hydroxy benzo[a]pyrene derivatives in chapter 5. Quantitative applications of lamp- and laser excited Shpol'skii spectrometry will be presented in sections 4.2 - 4.5 for parent PAHs and in chapters 5 and 7 for PAH metabolites.

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CHAPTER 3

SYNCHRONOUS FLUORESCENCE SPECTROSCOPY

3
SYNCHRONOUS FLUORESCENCE SPECTROSCOPY

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) usually show strong fluorescence emission, and a variety of fluorescence-based techniques has been applied to PAH analysis. As pointed out in the previous chapter, fluorimetry is a sensitive tool, suitable for the determination of pure compounds at low concentrations, but analysis of mixtures is usually hampered by interferences. Fluorescence emission spectra of PAHs consist of a number of relatively broad bands (typical band widths 300-1000 cm^{-1}, which corresponds to 5-15 nm), that cover a broad spectral range of up to 100 nm. In order to facilitate the proper quantitation of spectrally overlapping compounds, fluorimetric detection is often coupled to some form of (chromatographic) separation. Apart from physical separation, several alternative techniques are available to reduce spectral interference. The line-narrowing effect that results from cooling the analyte in a suitable matrix to cryogenic temperatures has been described in detail in chapter 2. In this chapter, we will consider another technique that can be used to reduce spectral overlap between different compounds in a mixture. Although the method does not yield the kind of high-resolution spectra that can be obtained with low-temperature techniques, it can still be of great value as a rapid, generally applicable screening method for not overly complex mixtures.

The concept of synchronous fluorescence spectrometry (SFS) was first put forward by Lloyd (1971a,b), and further developed by Vo-Dinh (1978, 1981). In the present chapter we will first provide a theoretical background, necessary to understand the spectral simplification that can result from synchronous scanning. Secondly, instrumental aspects and practical limitations of the technique will be considered. Finally, some examples of analytical applications of the SFS method will be presented. The term synchronous fluorescence spectrometry (SFS) is used throughout this chapter, but it should be noted that many theoretical and practical aspects of the technique are equally valid for synchronous phosphorescence measurements.

THEORY

In conventional molecular fluorescence spectroscopy, two types of spectra are generally discerned: emission and excitation spectra. In order to record an emission spectrum, the compound is excited at a fixed wavelength, while the fluorescence intensity is measured as a function of the emission wavelength. At ambient or lower temperatures, fluorescence emission of virtually all PAHs takes place after relaxation of the excited molecule to the lowest vibrational state of the first excited singlet state S_1 (see the Jablonski diagram in chapter 2, Fig. 1). Thus, the shape of the emission spectrum is independent of the excitation wavelength and only reflects the S_1-S_0 energy difference, the vibrational levels of the S_0 ground state, and the respective

transition probabilities. The excitation wavelength only affects the absolute intensity of the emission spectrum.

Alternatively, an excitation spectrum can be recorded by measuring the fluorescence intensity at a fixed wavelength, while scanning the wavelength of the excitation light. The absolute intensity of the excitation spectrum may be influenced by the choice of the wavelength monitored, but the shape of the excitation spectrum is emission-independent and only reflects the transitions and transition probabilities from the lowest vibrational level of the S_0 electronic ground state to vibrational levels of excited singlet states (S_1 and higher).

In mathematical terms, we can describe an emission measurement as follows:

$$I_{em}(\lambda_{em}) = Kcl\phi\, E_{ex}(\lambda_{ex})\, E_{em}(\lambda_{em}) \qquad (1)$$

where I_{em} is the measured intensity, $E_{em}(\lambda_{em})$ is a wavelength-dependent function describing the emission spectrum (arbitrary dimensions), ϕ is the fluorescence quantum yield, l is the effective light path, c is the concentration of the analyte, and K is a constant factor that includes several instrumental parameters. In this case, the excitation function $E_{ex}(\lambda_{ex})$ is considered a constant. Vice versa, an excitation experiment can be described as:

$$I_{ex}(\lambda_{ex}) = Kcl\phi\, E_{em}(\lambda_{em})\, E_{ex}(\lambda_{ex}) \qquad (2)$$

in which $E_{em}(\lambda_{em})$ is constant.

If λ_{ex} and λ_{em} are both variables, we obtain the total luminescence intensity function I_{TL}:

$$I_{TL}(\lambda_{ex}, \lambda_{em}) = Kcl\phi\, E_{ex}(\lambda_{ex})\, E_{em}(\lambda_{em}) \qquad (3)$$

The total luminescence intensity function is a multiplication of the two independent functions E_{ex} and E_{em}. It constitutes a three-dimensional hypersurface, that can be visualized as a pseudo-three dimensional stack plot or, alternatively, as a contour plot projected onto the λ_{ex}-λ_{em} plane, in which the contour lines connect points of equal intensity I (Fig. 1). A vertical cross-section through the TL plot produces the conventional excitation spectrum (λ_{em}= constant), while a horizontal cross-section parallel to the λ_{em} axis yields the conventional emission spectrum.

We shall now use the total luminescence plot of Fig 1 to visualize the most important advantages of the synchronous scanning technique. One can easily imagine that different cross-sections through the TL surface can be obtained if λ_{ex} and λ_{em} are both allowed to vary during the experiment. Although modern spectrofluorimeters with software-driven, independent monochromators could offer an unlimited number of scanning combinations, the traditional approach, as described first by Lloyd (1971a,b), involved a spectrofluorimeter in which the excitation and emission monochromators were mechanically interlocked, such that $\lambda_{ex} - \lambda_{em}$ = constant. The fluorescence intensity is recorded as the excitation wavelength trails the plotted

emission. Lloyd called the spectra obtained this way "synchronously excited fluorescence emission spectra", although they could also be regarded as excitation spectra with synchronously recorded emission (Vo-Dinh, 1978). In Fig. 1, the SFS spectrum is represented by a diagonal cross-section through the TL surface, along the line $\lambda_{em} - \lambda_{ex} = \Delta\lambda$ = constant:

$$I_{SFS}(\lambda_{ex}) = Kcl\phi\, E_{ex}(\lambda_{ex})\, E_{em}(\lambda_{ex} + \Delta\lambda) \qquad (4)$$

When the selected value for $\Delta\lambda$ is rather small, the SFS line slices through a corner of the TL plot, which results in an SFS spectrum that covers no more than a few nanometers. Fluorescence spectra can sometimes be reduced to a single narrow band if $\Delta\lambda$ is chosen to match the Stokes' shift of the analyte in the particular solvent (for PAHs typically 3-6 nm, (Vo-Dinh 1978)). The wavelength offset $\Delta\lambda$ can not be chosen too small because of Rayleigh scattering at $\lambda_{em} = \lambda_{ex}$. Obviously, spectral reduction leads to loss of information, but is at the same time one of the main advantages of the SFS method. Reduction of spectral overlap allows the determination of individual PAHs in complex samples, as was demonstrated by Vo-Dinh and Martinez (1981).

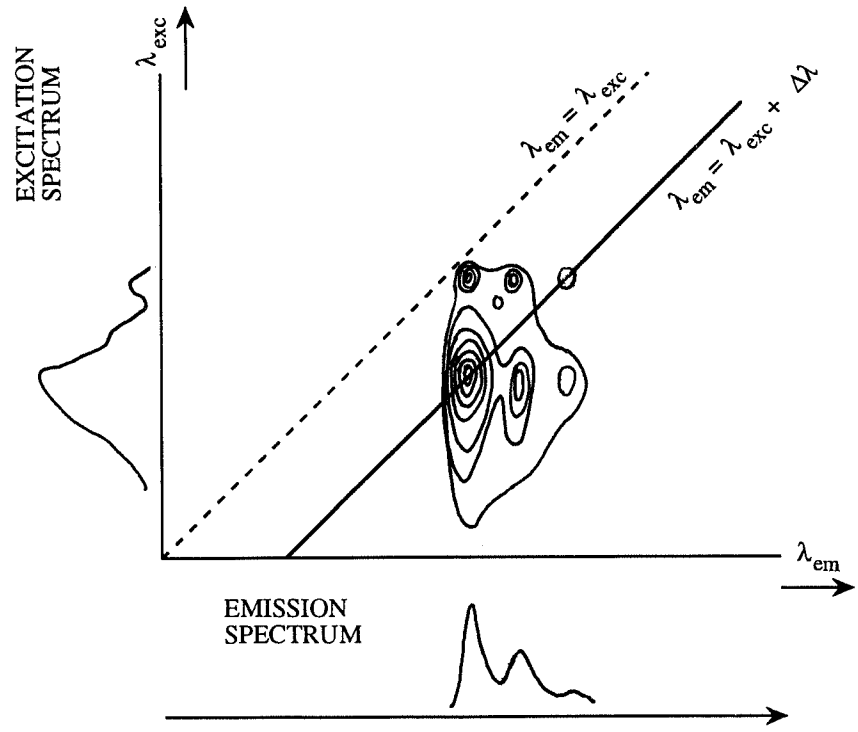

Fig. 1 Contour plot of total excitation-emission function of compound showing typical PAH vibrational structure. The synchronous spectrum is represented by the diagonal cross-section $\lambda_{em} = \lambda_{ex} + \Delta\lambda$. Rayleigh scattering occurs at $\lambda_{em} = \lambda_{ex}$.

Especially if the 0-0 band of the excitation and/or emission spectrum is not very intense (e.g. pyrene, benzo[a]pyrene), one may prefer to select a larger $\Delta\lambda$ and thus obtain a better sensitivity (Vo-Dinh and Martinez, 1981). In that case, the resulting SFS spectrum will stretch out over a spectral range of approximately $\Delta\lambda$, but may still be much simpler than the conventional fluorescence spectrum. In a conventional emission measurement, the complete spectrum is recorded using the same optimal excitation wavelength ($E_{ex}(\lambda_{ex})$ is constant in eqn. 3). In an SFS measurement, when the wavelength offset is chosen to match the difference between the maxima of excitation and emission, only the emission maximum will be recorded under optimal excitation conditions and with optimal sensitivity. The rest of the spectrum may not be completely removed, but will at least be less efficiently excited. $I_{SFS}(\lambda_{ex})$ is the product of two functions $E_{ex}(\lambda_{ex})$ and $E_{em}(\lambda_{ex} + \Delta\lambda)$; if $\Delta\lambda$ is properly chosen, these functions reinforce each other at one point and weaken each other at most other points of the spectrum. Figure 2 presents the conventional and SFS spectra of the PAH metabolite pyrene-1-glucuronide, using $\Delta\lambda$ = 37 nm. The absolute intensity of the SFS peak is equal to that of the conventional emission maximum (same excitation and emission maxima, same slit widths), but the spectrum is confined to approximately 37 nm and the intensity of the vibronic emission bands is strongly reduced.

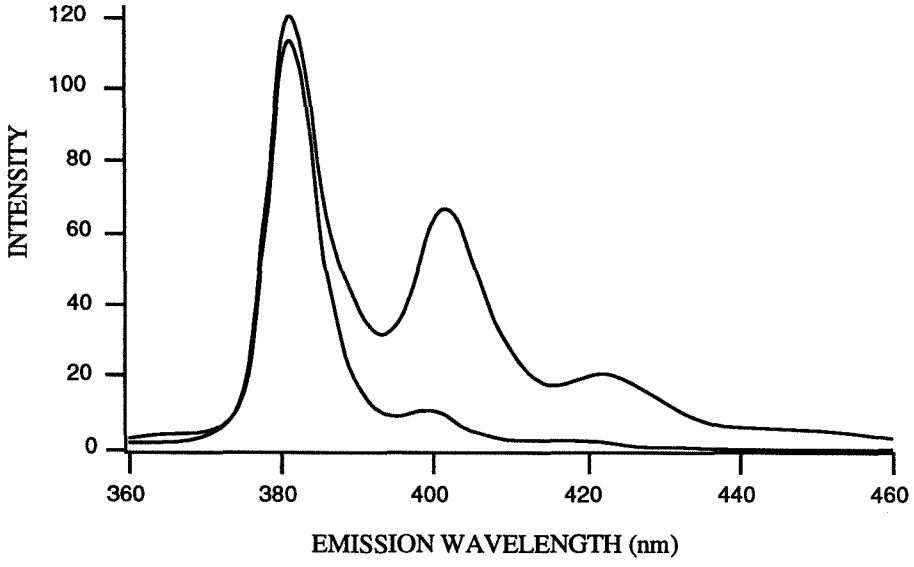

Fig. 2 Conventional (top; λ_{ex} = 345 nm) and synchronous (bottom; $\Delta\lambda$ = 37 nm) fluorescence spectra of 5 x 10^{-8} M pyrene-1-glucuronide in ethanol/water 50:50; intensities are plotted on the same scale; spectral band passes were 5 nm in both experiments.

The same effect may also lead to line-narrowing: when the excitation and emission bands have very different band widths, the band width of the resulting SFS peak will reflect that of the narrowest. If the excitation and emission bands have approximately equal widths, the resulting SFS peak will be narrower, since the edges of the emission are less optimally excited than the top.

Mathematically, we can illustrate this point if we assume the excitation and emission bands to have Gaussian profiles (Taylor and Patterson, 1987), described by the following functions:

$$E_{ex}(\lambda_{ex}) = K_1 e^{-(\lambda_{ex,max} - \lambda_{ex})/2\sigma^2_{ex}} \quad (5)$$

$$E_{em}(\lambda_{em}) = K_2 e^{-(\lambda_{em,max} - \lambda_{em})/2\sigma^2_{em}} \quad (6)$$

where $\lambda_{ex,max}$, $\lambda_{em,max}$ are the wavelengths of the excitation and emission maxima, and σ_{ex}, σ_{em} are the corresponding standard deviations. Assuming further that $\sigma_{ex} = \sigma_{em}$ (equal band widths), and substituting $\lambda_{em} = \lambda_{ex} + \Delta\lambda$ and $\lambda_{em,max} = \lambda_{ex,max} + \Delta\lambda$, the resulting product function is given by:

$$I_{SFS}(\lambda_{ex}) = K_3 c l \phi e^{-(\lambda_{ex,max} - \lambda_{ex})/\sigma^2_{ex}} \quad (7)$$

which describes a Gaussian function around excitation wavelength $\lambda_{ex,max}$, with a standard deviation $\sqrt{2}$ times smaller than the original functions.

EXPERIMENTAL ASPECTS

Nowadays, a synchronous scanning option is provided by most commercially available fluorimeters. Not all instruments, however, have monochromator entrance- and exit slits that can be adjusted to the low values (typically 1 nm resolution) necessary to obtain single SFS peaks at the 0-0 transition.

Ordinary quartz cuvettes can be used for the SFS measurements; smaller cuvettes (short path lengths) are preferred if the available sample volume is limited, or if matrix absorption effects have to be reduced. Depending on the geometry of excitation and collection optics of the particular instrument, the use of smaller cuvettes does not necessarily lead to a significant loss of sensitivity.

Matrix absorption effects, nonlinearity of response at high concentrations, excimer/exciplex formation, and dynamic quenching are potential confounding factors that must be considered whenever SFS measurements are carried out for quantitative purposes. The same problems are encountered in conventional fluorimetry, and most difficulties can be overcome by sufficient dilution, proper solvent choice, and deoxygenation.

Scattered light can be an important problem in SFS measurements. Raman scattering is

usually rather weak and can be adequately corrected for by simple subtraction of the solvent blank spectrum. Rayleigh scatter, however, is of an other magnitude and inhibits the recording of SFS spectra close to the $\lambda_{em} = \lambda_{ex}$ diagonal. For compounds with a strong 0-0 transition, a small wavelength offset, corresponding to the compound's Stokes' shift, is optimal and leads to the most rigorous spectral simplification. In order to reject Rayleigh scatter, the spectral band widths should be smaller than half the selected value for $\Delta\lambda$. Since the total light throughput (measured fluorescence intensity) is proportional to the fourth power of the spectral band width (two slits for each monochromator), small values of $\Delta\lambda$ can only be chosen at the expense of sensitivity. Adjusting $\Delta\lambda$ to the small Stokes' shift of the analyte is therefore only appropriate for the analysis of compounds at relatively high concentrations in very complex mixtures, when some sensitivity may be traded off against an improvement in selectivity (Vo-Dinh, 1981).

If larger wavelength offsets, and therefore wider monochromator slit widths, can be used, the absolute sensitivity of SFS is equal to that of conventional fluorimetry (see Fig. 2). In the absence of interferences, good fluorophores can be detected at the 10^{-10} M level (Uziel et al, 1987).

ANALYTICAL APPLICATIONS

Application of the SFS technique to a variety of analytical problems has been described in the literature. The method was used:

1) qualitatively, to obtain a fingerprint of complex mixtures
2) qualitatively, to identify one particular compound in a mixture
3) quantitatively, to determine one particular compound in a mixture and reduce interferences
4) quantitatively, to determine several compounds in a mixture

Some practical examples will now be briefly discussed to illustrate the usefulness of the SFS method in analytical chemistry.

To our knowledge, the SFS technique was first described by Lloyd (1971a,b), who developed the method for forensic purposes. Using various wavelength offsets, highly structured spectra were obtained for samples of used engine oil, whereas only a featureless broadbanded emission was observed when fixed excitation wavelengths were applied. The SFS spectra can be of great evidential value, for instance when traces of oil or grease are found at scenes of crime or at the site of a traffic accident. The same method was used to obtain fingerprint spectra of rubber material from tyre prints (Lloyd 1975), or to identify the source of crude oils in the case of illegal oil spillage at sea (John and Soutar, 1976).

In a recent paper by Manchester et al. (1990), the SFS method was used to study adducts of benzo[a]pyrene (BaP) metabolites to placental DNA. After acid hydrolysis at 90 °C, free metabolites were extracted and analyzed by means of SFS, using stepwise increasing values of $\Delta\lambda$. Specific peaks in the SFS spectra could be attributed to 7,8,9,10-tetrahydroxy tetrahydro BaP (BaP tetrol), but many unidentified fluorescent peaks were observed, suggesting that a

variety of compounds had formed DNA adducts.

Uziel and coworkers (1987) investigated the applicability of SFS to determine PAH metabolites in urine samples. BaP tetrol was chosen as a model compound. Direct determination in untreated urine was not possible, owing to various matrix effects; a dilution 1:100 was necessary to restore the normal fluorescence properties of the analyte. BaP tetrol could be determined in rat urine, 24 hours after injection with 0.2 mg BaP. The limit of detection in blank urine spiked with BaP tetrol was 30 ng/ml. Application to human studies was not reported.

In chapter 6 of this thesis, the SFS method is used to quantitate pyrene-1-glucuronide in fish bile samples from more or less polluted areas. Bile of fish captured in the field contains a very complex mixture of different PAH metabolites, but pyrene-1-glucuronide is one of the major components (Krahn et al., 1987) and is therefore easily quantitated with SFS. For this particular problem, the main advantage of synchronous scanning is spectral simplification (see Fig. 2). The detection limit in bile was 10-20 ng/ml, using 200-fold dilution.

Vo-Dinh and Martinez (1981) have demonstrated the applicability of the SFS technique to the quantitation of PAHs in a solvent refined coal sample. Anthracene, benzo[b] fluorene, benzo[a] pyrene, fluoranthene, fluorene, perylene, and pyrene were determined in the sample; no sample treatment was applied. For most compounds $\Delta\lambda = 3$ nm was chosen; larger offsets were necessary for benzo[a]pyrene, pyrene, and fluoranthene (low 0-0 absorption). The analysis was carried out as part of an intercomparison exercise, and yielded quite satisfactory results in comparison with the reference values listed by the U.S. National Bureau of Standards. Inman and coworkers (1982) applied synchronous scanning to a PAH mixture in a Shpol'skii matrix at 77 K (using a constant energy difference of 1400 cm^{-1}), and observed a considerable spectral simplification compared to conventional Shpol'skii spectra.

CONCLUSIONS

Synchronous fluorescence spectrometry is a useful and versatile technique for the qualitative and quantitative analysis of fluorescent analytes, such as PAHs or PAH derivatives. The method is very fast and does usually not require extensive sample preparation. SFS spectra can be recorded with ordinary commercial spectrofluorimeters, and even direct measurements in the field are possible (Eastwood et al., 1993).

Although the SFS method should not be expected to be as selective as Shpol'skii spectrometry or GC-MS, it can be very useful as a first screening method, or if only a few dominant compounds are to be determined. Rapidity and low cost are especially important if large numbers of samples are to be analyzed (see section 6.2). Although SFS peaks in complex mixtures may not be completely resolved, accuracy and precision of the method may still be acceptable in comparison with more advanced analytical techniques that require delicate instrumentation or several sample pretreatment steps (Vo-Dinh and Martinez, 1981; see also section 6.1).

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CHAPTER 4

SHPOL'SKII SPECTROFLUORIMETRIC ANALYSIS OF PARENT PAHs

4.1

SHPOL'SKII FLUORIMETRY AS AN INDEPENDENT IDENTIFICATION METHOD TO UPGRADE ROUTINE HPLC ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

High Performance Liquid Chromatography combined with fluorescence detection is routinely used in the Dutch Water Quality Survey to determine the Polycyclic Aromatic Hydrocarbons (PAH) contents of marine sediment samples. In this paper, Shpol'skii spectroscopy is utilized to ascertain the identity and the purity of the peaks in the chromatogram by collection of several eluting fractions and subsequent spectroscopic analysis. The low temperature Shpol'skii technique provides high-resolution fluorescence spectra of PAHs that can serve as fingerprints. Thus, information concerning peak purity was obtained and the number of components identified was roughly doubled.

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are among the most common pollutants of our environment. Some PAHs (e.g. benzo[a]pyrene, dibenz[ah]anthracene) are strongly carcinogenic and constitute a threat, even if present at low concentrations. Thus, it is important to determine their presence in, for example, cigarette smoke, urban air, food and environmental samples.

A major analytical problem in the determination of PAHs is the separation and identification of individual components in the presence of many isomers and alkylated compounds that have very similar physical properties. Both liquid- and gas chromatography are applied to the analysis of PAHs in environmental samples (Vo-Dinh and Winefordner, 1989). For the detection of PAHs in reversed-phase HPLC, their native fluorescence can be used to reach very low detection limits. However, the identification power of reversed-phase HPLC with fluorescence detection is limited. Since the excitation and emission wavelengths are not specific enough to distinguish between closely related compounds, identification has to be done mainly on the basis of retention time and, furthermore, peak purity cannot be sufficiently ascertained. Therefore, it was decided to analyse a number of fractions from the chromatogram with an independent identification method.

Shpol'skii spectroscopy is especially suited for the qualitative analysis of PAHs at trace levels, as it combines the sensitivity inherent to fluorescence methods with the specific information that can be obtained in infrared spectroscopy (Hofstraat et al., 1985; Garrigues et al., 1987). It makes use of frozen n-alkane matrices at cryogenic temperatures to reduce band broadening which is the cause of the limited identification power of room temperature fluorescence. The PAHs occupy substitutional sites in the n-alkane polycrystalline matrix, one guest molecule replacing one or more n-alkane molecules, resulting in largely identical surroundings (Nakhimovsky et al., 1989). The appearance of the Shpol'skii spectrum may vary if different n-alkanes are used, as the fit of the PAH within the crystal is critical. A Shpol'skii spectrum consists of a number of narrow lines (vibrational fine structure) with a full width at half maximum (FWHM) of 0.1- 0.01 nm. These lines are suitable for identification purposes because they form a fingerprint of the individual PAHs. Shpol'skii spectroscopy cannot be used for on-line detection in HPLC, for the solid matrix precludes compatibility with flow systems.

In this investigation, suspended matter samples from the Western Scheldt (south-west of the Netherlands) were taken and PAHs were determined with HPLC/fluorescence detection, using a routine analytical method developed at the Tidal Waters Division of the Ministry of Transport and Public Works. A total of 21 separate fractions of the chromatogram was collected and analyzed by Shpol'skii spectroscopy at the Department of General and Analytical Chemistry of the Free University Amsterdam. The main goal of this study was the determination of peak purity and the identification of unknown components.

EXPERIMENTAL

Sample clean up and chromatography prior to Shpol'skii spectroscopy

Suspended matter was collected by centrifugation at three different stations in the Western Scheldt. The samples were pooled and freeze-dried, homogenized and extracted with n-hexane/acetone (3:1,v/v) in a Soxhlet apparatus for 4 hours at 80 °C. After replacing the solvent with 1 ml of iso-octane the extract was cleaned over an Al_2O_3/Na_2SO_3 column (elution with n-hexane). The eluent was concentrated to 1 ml and transferred to a SiO_2 column (2 gram, dried at 180 °C). The PCB-fraction was eluted with 25 ml of n-hexane; afterwards, the column was dried with nitrogen and PAHs were subsequently eluted with methanol.

For HPLC separation, a HP 1090 system was employed with a HP 1046A fluorescence detector, controlled by a HP 9000 computer (all from Hewlett- Packard). Separation was carried out using a Vydac 201TP5 250*4.6 mm i.d. reversed-phase column by applying the following gradient elution program: water-methanol 30/70 at t = 0; rapid change to 80/20 at t = 5 min; gradual change to 100 % methanol at t = 50 min; return to start-up at t = 90 min. The flow rate was 0.9 ml/min. Benzo[b]chrysene was added as an internal standard for the HPLC-analysis as recommended by the Marine Chemistry Working Group of the International Council for the Exploration of the Sea (ICES). Although the Hewlett-Packard system allows for programmed variation of excitation and emission light, in this study a fixed excitation wavelength of 250 nm was applied (band pass 12.5 nm) and total emission was detected in 0th order. A 300 nm cut-off filter was used in front of the emission slit to remove excitation light.

After having passed the fluorescence detector, a number of fractions was collected. The solvent was replaced by n-hexane, and the fractions were further analyzed by means of low-temperature Shpol'skii spectroscopy.

In a separate HPLC run, additional information was obtained by recording on-line fluorescence spectra: the flow was stopped at regular intervals (0.3 min) and an emission spectrum (300-500 nm, 3nm/sec) was taken. If a substantial emission was detected also the excitation spectrum (215-335 nm, 6 nm/sec) was recorded. In some cases, the on-line room-temperature spectra gave a first indication of the identity of the compound concerned. During the elution of a peak, several spectra were taken; dissimilarities between these spectra point at peak impurity.

Shpols'kii spectroscopy

Samples were contained in a gold-plated copper sample holder, covered by a sapphire window, and fitted to the cold tip of a CTI Cryogenics (Waltham, MA) model 20 helium refrigerator. For excitation, a 450 W xenon arc lamp and a Bausch and Lomb (Rochester, NY) monochromator (band pass 8 nm FWHM) were used. Stray light from the excitation monochromator was rejected by using several band pass filters. Samples were illuminated front-face. Fluorescence was collected by a 10 cm F/1 lens, passed through a cut-off filter, and

focussed on the entrance slit of a Jobin-Yvon (Longjumeau, France) HR1000 monochromator (resolution 0.16 nm FWHM). The light was detected with a cooled photomultiplier tube (type C31034-A2, RCA, Lancaster, UK) operated at 1500 V. The detector signal was further processed by a home-built preamplifier and a Stanford Research (Palo Alto, CA) SR400 photon counter. Spectra were recorded with a Kipp & Zonen BD8 chart recorder (Delft, The Netherlands).

Initially, it was examined whether a Shpol'skii spectrum could be obtained using frozen n-hexane (28 K). Subsequently, the solvent was replaced by n-octane and a second measurement was attempted with that matrix.

RESULTS AND DISCUSSION

Fig. 1 depicts the HPLC chromatogram applying fluorescence detection. The compounds identified prior to Shpol'skii analysis and the 21 collected fractions are indicated.

The identification power of Shpol'skii spectroscopy for PAHs is illustrated by figure 2, showing both the on-line recorded room-temperature (RT) and the Shpol'skii spectra of fraction IX. On the basis of retention time, fraction IX was identified as benzo[b]fluoranthene (B[b]F), but the on-line RT spectra (recorded at different positions of the eluting peak) indicate that at least one other compound is present. A conclusive identification, however, could not be derived from these spectra. The Shpol'skii spectra (Fig. 2c,d) clearly show that the second compound in fraction IX is perylene.

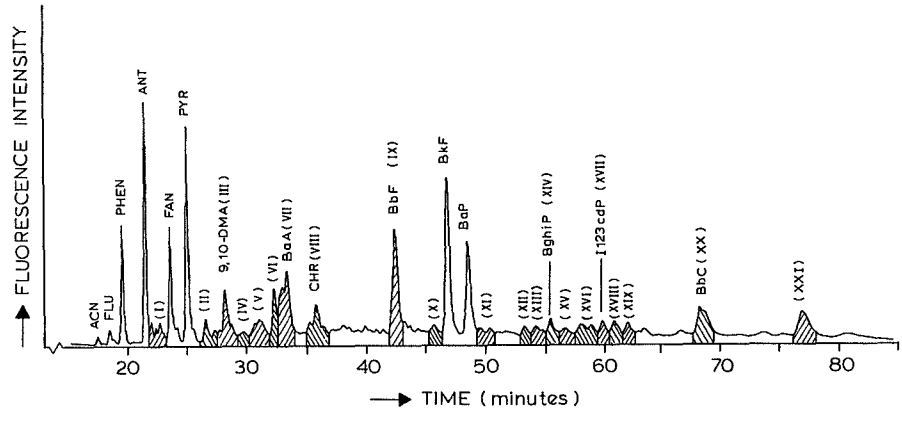

Fig. 1 Reversed-phase HPLC chromatogram of suspended matter sample; ACN = acenaphtene; FLU = fluorene; PHEN = phenanthrene; ANT = anthracene; FAN = fluoranthene; PYR = pyrene; 9,10-DMA = 9,10-dimethylanthracene; BaA = benz[a]anthracene; CHR = chrysene BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BaP = benzo[a]pyrene; BghiP = benzo[ghi]perylene; I123cdP = indeno [1,2,3-cd] pyrene; BbC = benzo[b]chrysene.

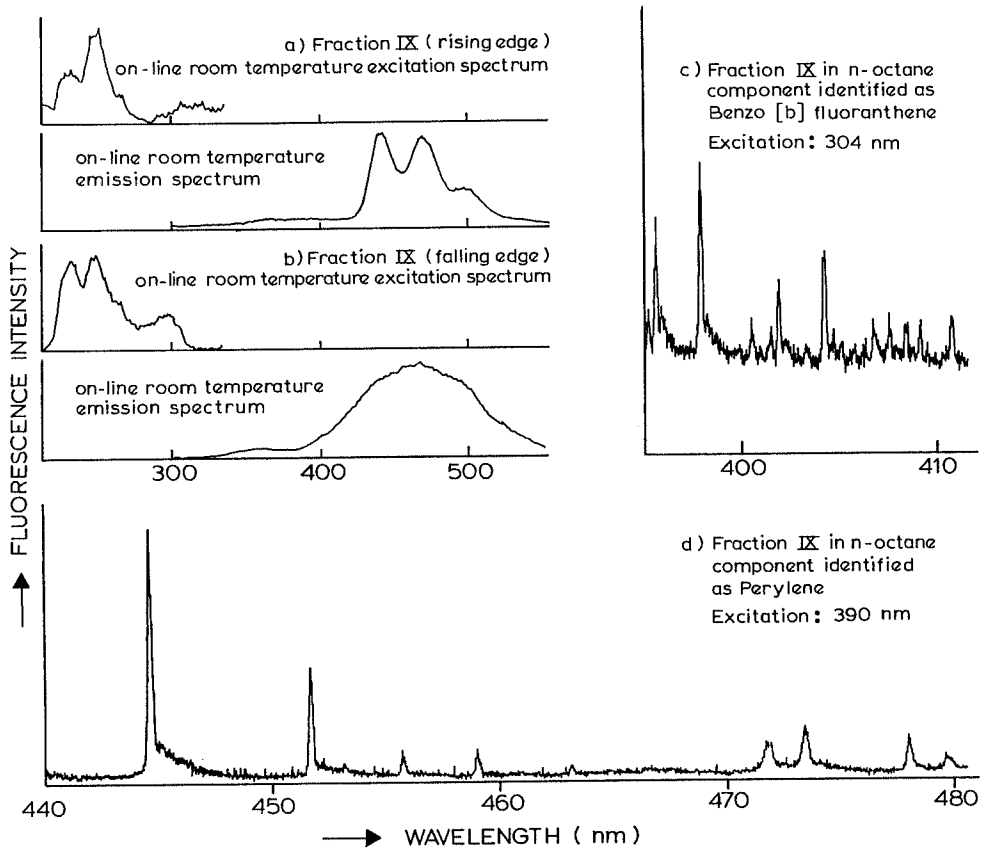

Fig. 2 On-line room temperature spectra (a,b) and off-line Shpol'skii spectra (c,d) of fraction IX (Shpol'skii matrix n-octane).

Neither the chromatographic peak shape (Fig. 1), nor the room-temperature spectra gave any indication that fraction XV contained more than one component. The Shpol'skii spectra, however, show that not only dibenz[ah]anthracene (DB[ah]A) is present, but also some unidentified components (see Fig. 3). Between the emission lines of DB[ah]A (marked with *), several other peaks can be distinguished. The spectra illustrate that, because of the line-narrowing effect obtained with Shpol'skii spectroscopy, even compounds emitting in the same wavelength region will seldomly show spectral overlap.

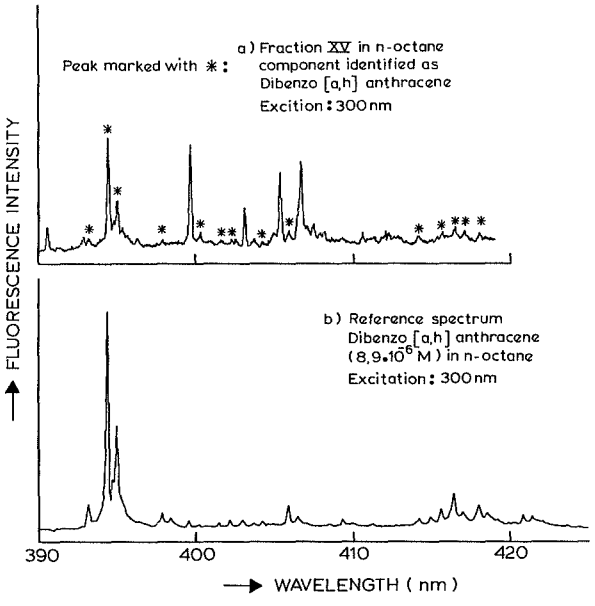

Fig. 3 Shpol'skii spectrum of fraction XV in n-octane (2a) and of dibenz[ah]anthracene standard solution (8,9 x 10^{-6} M) (2b). The peaks marked with * are attributed to DB[ah]A.

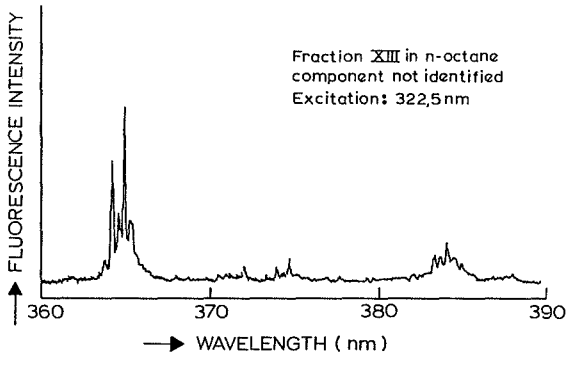

Fig. 4 Shpol'skii spectrum of fraction XIII in n-octane; compound not identified.

Identification was achieved by comparison with reference spectra. Unfortunately, the number of available reference spectra is still limited (Nakhimovsky et al., 1989; Karcher et al., 1983). Often, a Shpol'skii spectrum was obtained from a certain fraction, but no matching reference could be found. This is illustrated by the spectrum of fraction XIII in Fig. 4 and by the unidentified peaks in the spectrum of fraction XV (Fig. 3).

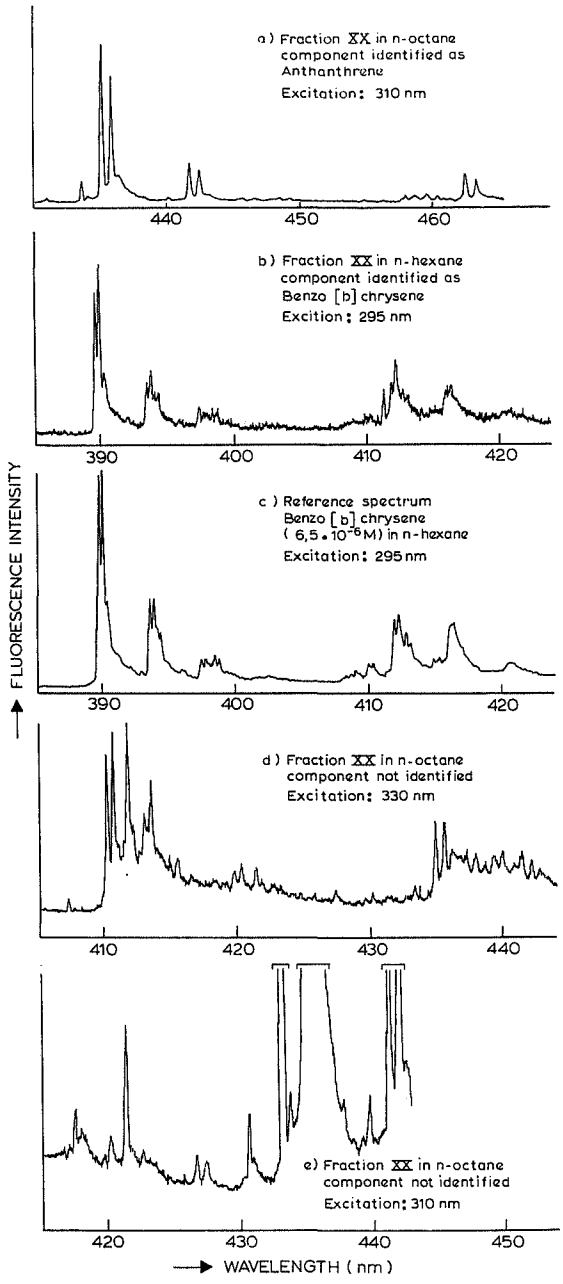

Fig. 5 Shpol'skii spectra of fraction XX (5a, d, e in n-octane; 5b in n-hexane) and of benzo[b] chrysene standard solution (6,5 x 10^{-6} M in n-hexane) (5c). The strong emission lines in Fig. 5e are attributed to anthanthrene.

The composition of fraction XX is of special importance, since it contains benzo[b]chrysene, B[b]C, added as an internal standard. Obviously, it is crucial to measure such a standard without interferences. The high-resolution spectra, obtained from fraction XX, are shown in figure 5, together with a reference spectrum of B[b]C. Fraction XX does not only contain benzo[b]chrysene but at least three other species are present. One of the compounds that coelute with B[b]C could be identified as anthanthrene (ATT), which provides a comparably high signal. The interference of ATT can be reduced, but not completely removed, under room temperature conditions by using an excitation wavelength of 280 nm and an emission wavelength of 390 nm for B[b]C. The other unidentified components of fraction XX show emission maxima much nearer to that of benzo[b]chrysene but give rise to a lower fluorescence intensity. The excitation maxima of these components (under Shpol'skii conditions) are 330 and 310 nm, their emission maxima are ca. 410 and 420 nm, respectively (Fig. 5d,e). (We made sure, with the help of excitation spectra, that the peaks in Fig. 5e do not belong to any of the other three constituents of fraction XX). One should be careful applying Benzo[b]chrysene as an internal standard for HPLC/fluorescence purposes, as it will probably be impossible to remove all interferences spectroscopically.

The results of the qualitative analysis of the 21 fractions are summarized in Table I. Compounds identified in the present study are underlined; an asterisk indicates that a Shpol'skii spectrum was obtained but that no matching reference spectrum was available. The latter aspect underlines the potential of the method: once a larger library of reference spectra is available, the identification possibilities of the Shpol'skii method will be greatly enhanced.

Table I Polycyclic Aromatic Hydrocarbons in suspended matter sample identified with Shpol'skii spectroscopy.

Fraction	PAH	Fraction	PAH
I	4,5-Methylenephenanthrene	XIII	*
II	Triphenylene	XIV	Benzo[ghi]perylene
V	*	XV	Dibenz[ah]anthracene; *; *
VI	Benzo[b]fluorene	XVI	*
VII	Benz[a]anthracene; 2-Methylpyrene; *	XVII	Indeno[1,2,3-cd]pyrene
VIII	Chrysene; *	XVIII	*; *
IX	Benzo[b]fluoranthene; Perylene	XIX	*
X	Dibenz[ac]anthracene; *; *	XX	Benzo[b]chrysene; Anthanthrene; *; *
XI	Dibenz[aj]anthracene * *	XXI	Dibenzo[bk]fluoranthene; *

* Compound showed quasilinear spectrum, but could not be identified. Compounds underlined were identified in this study. No Shpol'skii spectra were obtained from fractions III, IV and XII.

However, even without reference spectra, the range of possible PAHs can be narrowed down considerably by taking advantage of the appearance and the emission wavelengths of the Shpol'skii spectrum. An example is the spectrum of fraction XIII (Fig. 4), which is chrysene-like, but which does not match with the spectra of chrysene or one of the methylchrysene isomers. The compound(s), however could very well be a C2-substituted chrysene, as these can be present in significant quantities in sediment samples according to HPLC-MS measurements (Quilliam and Sim (1988).

From the above it is clear that Shpol'skii spectroscopy can be used for peak purity assessment in HPLC and for identification purposes. The number of identified compounds was roughly doubled by the present investigation. Comparing the Shpol'skii technique with mass spectrometry as an identification method for PAHs in combination with HPLC, MS has a great advantage because it can relatively easily be coupled in an on-line mode. Shpol'skii spectroscopy, on the other hand, offers better sensitivity and more spectral information. This is essential for the identification of isomeric compounds which are often difficult to distinguish with mass spectrometry, as the mass spectra of PAHs usually show the molecular ion only. With the Shpol'skii technique, isomers in general give well distinguishable spectra. The Shpol'skii spectra of benzo[b]fluoranthene and perylene, which are of the same molecular weight and elute at the same time, are shown in Fig. 2. Even methyl substituted isomers show significantly different spectra (Garrigues et al., 1987). At the moment, the bottleneck is the non-availability of reference spectra. Especially spectral data of alkyl-substituted PAHs, that seem to be relatively abundant in areas exposed to petrochemical pollution (Quilliam and Sim, 1988), are still scarce. An obvious disadvantage of the Shpol'skii technique is that it can only be practised in an off-line mode. We would like to emphasize, however, that the analysis of the separate eluting fractions needs only be carried out once. The results of this procedure can be used for a (qualitative) validation of the routine HPLC-analysis.

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4.2

QUANTITATIVE SHPOL'SKII ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS IN STANDARD SOLUTIONS: AN INTERCOMPARISON EXERCISE

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ABSTRACT

Eight polycyclic aromatic hydrocarbons (PAHs) were determined in standard solutions by means of lamp-excited Shpol'skii spectrofluorimetry at 28 K in n-octane matrices. The analysis was carried out within the framework of an intercomparison exercise organized by the International Council for the Exploration of the Sea (ICES). During the exercise, some aspects of the measurement protocol (concentration of internal standard, optical resolution, storage of stock solutions) were identified as potential sources of error. The improved procedure yielded excellent quantitative results: average accuracy was 2.5 %; average precision 4.2 %.

INTRODUCTION

At its 1987 meeting in Copenhagen, the marine chemistry working group of the International Council for the Exploration of the Sea (ICES) decided to start a fourth hydrocarbon intercomparison exercise. Results of previous intercomparison exercises had revealed that the determination of specific PAHs in marine environmental samples (seawater, suspended matter, sediments and biota) still poses analytical problems. It was decided that a stepwise approach, stretching out over several years, should be followed to improve interlaboratory consistency. A group of ten non-alkylated PAHs with 3-6 fused rings was selected, covering a wide range of polarities or boiling points. Other selection criteria were environmental significance and availability as certified pure compounds. Especially those compounds were included that had caused specific problems during the earlier exercises, as the result of photolability, and/or evaporation loss.

The first stage of the exercise in 1989 was mainly intended for instrument calibration: a standard solution of the PAHs at unknown concentration was sent to all participating laboratories, together with a similar solution with known concentrations for calibration, as well as a solvent blank. The solutions were distributed in flame-sealed ampoules; separate solutions were prepared for GC analysis (in hexane), and for HPLC analysis (in acetonitrile).

17 Laboratories took part in the exercise, some applying more than one technique. 14 Sets of gas chromatographic results were returned (7 using flame ionization detection (GC-FID); 7 using mass spectrometric detection (GC-MS)), and 8 sets of HPLC data (5 using fluorescence detection (HPLC-Flu); 3 using UV absorption detection (HPLC-UV)). Here, we report on the analysis of the standard solution in hexane, using Shpol'skii fluorescence spectrometry. After some improvements in methodology and instrumental setup, a second determination of the same solution was carried out in August 1990.

EXPERIMENTAL

Sample preparation

Ampoules were stored in the dark at -20 °C, and opened when still cold to diminish the risk of solvent evaporation. The unknown standard solution G2 was diluted with an equal volume of a 1.0×10^{-3} M solution of perdeuterated pyrene in n-octane (Baker analyzed grade), and subsequently diluted 1:500 with n-octane. This way, the final analytical solution contained the PAHs at unknown concentrations of approximately 20-100 ppb, and pyrene d_{10} at $1{,}0 \times 10^{-6}$ M. The internal standard allowed for correction of several experimental factors, e.g. optical alignment, sample thickness, evaporation of solvent. The dilutions of the calibration solution G1 were prepared in a similar manner.

For the second determination, the samples were stored at 4 °C, and first allowed to reach room temperature in an ultrasonic bath before opening, in order to redissolve PAHs adhered to the glass wall. This time, the internal standard was added at a lower concentration: 2.0×10^{-7} M.

Apparatus

10 µl samples were contained in a gold-plated copper sample holder, covered by a sapphire window, and mounted to the cold tip of a CTI Cryogenics Model 20 closed-cycle helium refrigerator (Waltham, Massachusetts, U.S.A.). Four samples could be cooled simultaneously to 28 K in 45 minutes. For excitation we used a 450 W xenon arc lamp and a Bausch & Lomb monochromator (Rochester, New York, U.S.A.; bandpass 8 nm full width at half maximum, FWHM). A 1 cm quartz cuvette with a 25% solution of nickel sulphate in water served as an extra band-pass filter; it rejects stray light very efficiently in the 370-440 nm region. For extra selectivity, each compound was measured separately using the optimal excitation wavelength. Fluorescence from the samples was collected at a 20° angle from the incident light by a positive lens, passed through a cut-off filter, and focussed on the entrance slit of a Jobin-Yvon HR1000 monochromator (Longjumeau, France) The resolution was 0.16 nm (1st series), or 0.10 nm (2nd series). The first series of measurements was carried out using an intensified diode array detector (Princeton Instruments IRY 1024 GRB). Data processing was carried out on a HP Vectra ES/12 computer, using the Princeton Instruments OSMA and PSMA software. For the second series of measurements a cooled photomultiplier tube (Philips XP2020Q), a Stanford SR 400 photon counter, and a Macintosh plus computer with home-written data acquisition software was used.

Analysis

With the diode array detector, a spectral range of 20 nm around the main emission line of each compound was recorded; the total exposure time was typically 10 s per spectrum. When a single detector was used, a spectral range of typically 3 nm around the main emission line was recorded step-by-step, employing a scanning rate of 0.01 nm/s and a detector integration time of 1s. For quantitation the peak area of the main emission line was divided by that of the internal standard pyrene d_{10}. With the xenon-lamp excitation source used in this study, detection limits ranged from 10^{-8} M for some weaker fluorophores (benzo[b]fluoranthene, benzo[e]pyrene, indeno[1,2,3-cd]pyrene) to 3×10^{-10} M (benzo[a]pyrene). At high concentrations (> 10^{-6} M), calibration curves tend to level off, owing to self-absorption. Earlier calibration plots had been shown to be straight from 10^{-6} M down to the detection limit; for this exercise only three dilutions were prepared, which extended over one decade. Every point of the calibration curves was measured at least three times, and six separate measurements of the unknown solution G2 were carried out.

Fluoranthene and phenanthrene do not produce good quasilinear spectra in an n-octane matrix. Quantitation of these compounds in n-hexane was not attempted.

RESULTS AND DISCUSSION

In (lamp excited) Shpol'skii spectroscopy, compatibility of the analyte with the matrix (no complex multiple-site spectra), combined with an optimal cooling regime (Hofstraat et al., 1989) is most important. Furthermore, for optimal sensitivity, the excitation source should possess a high spectral radiance at an absorption maximum of the particular compound. The analyte should not only have a high fluorescence quantum yield, but also a large fraction of the total spectrum should be concentrated in a single emission line. In complex mixtures, spectral overlap may become a problem; the possibilities to employ selective excitation are limited with a broad-banded excitation source.

The results of the first measurements (see Table I) show an excellent agreement with the nominal values for pyrene, benz[a]anthracene, benzo[a]pyrene and indeno[1,2,3-cd]pyrene. Although the emission of the latter is rather weak, it could still be accurately measured, since there were no interferences. The weak emission lines of benzo[b]fluoranthene and benzo[e]pyrene suffered from spectral overlap with vibrational bands of pyrene d_{10}, which led to problems with the quantitation of those two compounds. For the second analysis, a lower concentration of internal standard was added, and spectral overlap was further diminished by reducing the emission spectral band width to 0.1 nm. The results in Table I illustrate the improvement in accuracy for benzo[b]fluoranthene and benzo[e]pyrene.

The results of the first measurement also indicated a large discrepancy for chrysene and benzo[ghi]perylene, which could not be attributed to spectral interferences. Both compounds showed strong quasilinear emission, and could be determined without spectral overlap. Later, it was found that independent calibration plots for these two compounds had significantly steeper slopes than the curves prepared from the G1 calibration solution. We concluded that some loss of chrysene and benzo[ghi]perylene must have occurred during shipment or sample treatment, as we were informed that other laboratories had found concentrations much closer to the nominal values. We hypothesized that storage of the ampoules at -20 °C could have caused the more sparingly soluble PAHs to precipitate or adhere to the glass wall. The fact that the calibration solution G1 was opened and diluted when it was still cold, while the unknown solution was treated somewhat later after having reached room temperature, could probably explain the observed bias. A second set of standard solutions in sealed ampoules was first sonicated and allowed to reach room temperature before opening. The results in Table I indicate that the measurement accuracy for chrysene and benzo[ghi]perylene was strongly improved by this procedure.

In addition to the PAHs listed in Table I, solution G2 was also found to contain perylene (not quantitated), presumably added to the test mixture because it is difficult to separate from benzo[b]fluoranthene with most HPLC systems. In a Shpol'skii measurement, however, perylene causes no spectral overlap with any of the other PAHs of the test mixture.

Table I Measured and nominal values for 8 PAHs in n-hexane standard solution.

PAH	1st measurement	bias (%)	2nd measurement	bias (%)	nominal value
Pyr	63.6 ± 0.9	6.5	70.2 ± 1.4	+ 3.2	68
BaA	44.2 ± 1.0	6.0	47.0 ± 1.0	0	47
Chr	101.5 ± 5.0	+146	45.0 ± 2.3	0	45
BeP	55.1 ± 2.6	29	85 ± 4.4	+ 9.0	78
BaP	119.6 ± 3.2	+ 4.3	117.0 ± 3.5	+ 1.7	115
BbF	42.7 ± 3.1	+ 64	26.1 ± 1.1	+ 0.4	26
BgP	117.8 ± 4.6	+ 57	76.4 ± 2.3	+ 1.9	75
InP	52.2 ± 2.8	+ 1.5	55 ± 4.8	+ 3.8	53

Concentrations in µg/ml; results are arithmetic means of 6 determinations, ± standard deviations. Bias = deviation of the mean determined value (6 replicates) from the nominal value. Pyr = pyrene; BaA = benz[a]anthracene; Chr = chrysene; BeP = benzo[e]pyrene; BaP = benzo[a]pyrene; BbF = benzo[b]fluoranthene; BgP = benzo[ghi]perylene; InP = indeno[1,2,3-cd]pyrene.

Fig. 1 illustrates the overall performance of the various laboratories (full details in Law and Nicholson, 1993). The relative standard deviation and the relative bias (positive or negative difference between the average of 6 measurements and the nominal value) are averaged over all compounds analyzed. For most laboratories, the bias (systematic error) was higher than the standard deviation, which was also observed by Sim et al. (1987) during a similar intercomparison exercise organized by the National Research Council Canada. Fig. 1 reveals that large differences in analytical performance can occur between laboratories using the same technique. On the average, the four chromatographic methods showed similar accuracies (except for laboratory nr. 16); precision was generally better for HPLC. Of course, strengths and weaknesses of each method will become more clear when real sediment or biota samples are to be analyzed.

The results of the first Shpol'skii analysis showed reasonable standard deviations, but for some compounds, the accuracy was among the worst. However, the main causes of these errors were identified, and the second measurement yielded quite satisfactory results.

Unfortunately, the intercomparison exercise did not proceed at the intended pace, owing to organizational problems. For that reason, we have not been able to test the analytical performance of the Shpol'skii technique for real samples within the framework of the exercise. However, the applicability of the Shpol'skii method to the quantitative analysis of PAHs in sediment and biota samples will be demonstrated in the following sections.

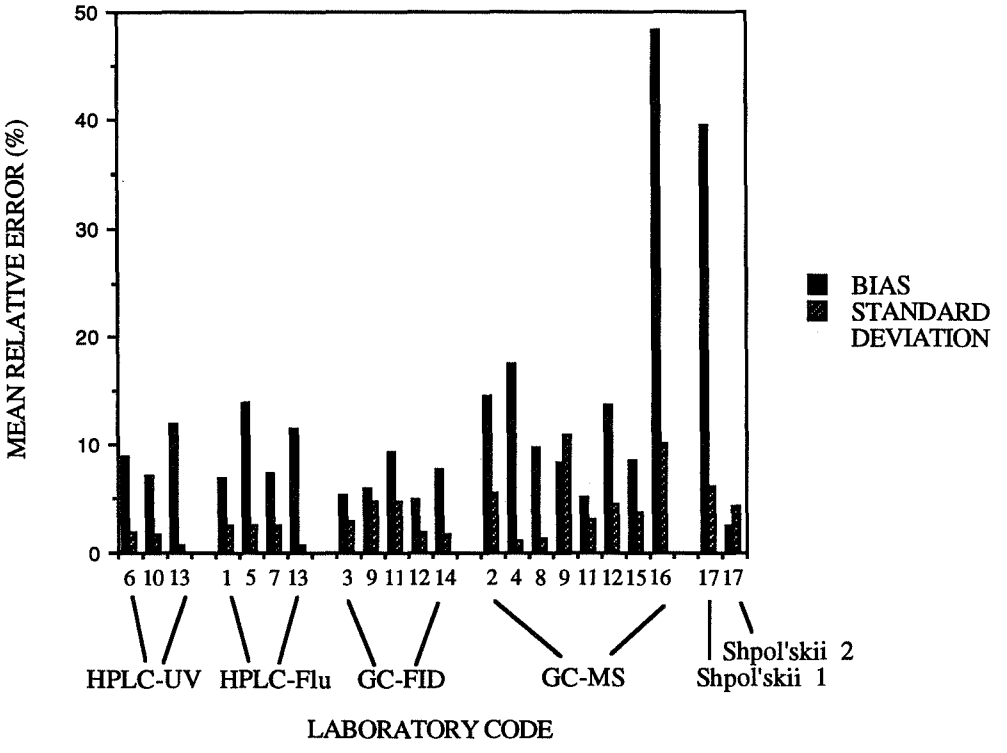

Fig. 1 Analytical performance of the participating laboratories, showing accuracy and precision (as percentage of the nominal value), averaged over all compounds analyzed. Bias = deviation of the mean determined value (6 replicates) from the nominal value.

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4.3

SHPOL'SKII SPECTROFLUORIMETRIC ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS IN SEDIMENT REFERENCE MATERIALS

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) were determined in sediment reference materials from the National Research Council Canada (NRCC), using lamp-excited Shpol'skii spectrofluorimetry at 28 K in n-octane matrices. The Shpol'skii analysis could be carried out on crude extracts; calibration plots and standard addition methods were used for quantitation. For comparison, the same extracts were analyzed using HPLC with fluorescence detection.
For the harbor sediment materials HS-4 and HS-6, both techniques yielded good results, reasonably close to the reference values, although a slight bias was apparent: for most compounds the mean values increased in the order: HPLC<Shpol'skii<NRCC. In the spiked reference material SES-1, however, concentrations of some compounds were found to be significantly lower than stated by the NRCC.

INTRODUCTION

Environmental pollution with polycyclic aromatic hydrocarbons (PAHs) may have serious adverse effects on human and animal health (Baumann, 1989). PAH concentrations in various environmental compartments (air, soil, water, sediment, biota) are regularly monitored by numerous analytical laboratories worldwide. Most analytical techniques are based on chromatographic separation: high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), or gas chromatography (GC). For the quantitation of PAHs, these separation techniques can be coupled to one or more of the following detection principles: UV absorption, fluorescence, mass spectrometry (MS), or flame ionization detection (FID).

When analyzing environmental samples, laboratories are usually able to determine most PAHs from the U.S. Environmental Protection Agency (EPA) priority pollutant list with a reasonable precision. Intercomparison exercises, however, revealed that accuracy is often much poorer (Sim et al., 1987; see also previous section). Large biases may exist between results obtained in different laboratories or between analytical data obtained with different techniques (Sim et al., 1987, 1988). These systematic errors may be related to the sample preparation (incomplete extraction, poor recoveries, photoreactivity, evaporation losses, etc.) or to the analytical method employed (poorly resolved peaks, matrix interferences, non-linearity of response). Although responsibility for the quality of the analytical data lies of course first with the experimentator, scientific organizations or regulatory institutions may help improving inter-laboratory or inter-method consistency by organizing intercomparison exercises or by the development of standard reference materials (Sim et al., 1988).

Shpol'skii spectrofluorimetry is an alternative technique for the determination of PAHs in complex samples. The advantages of the Shpol'skii method for the unambiguous identification of closely related compounds is generally recognized (see section 4.1, Mastenbroek et al., 1990; chapter 5, Ariese et al, 1993; Garrigues et al., 1985), but it was believed that specific features of the method would preclude quantitative applications (Lukasiewicz and Winefordner, 1972). Shape and intensity of the quasilinear Shpol'skii spectra may depend rather critically on various experimental parameters, such as the type of matrix selected, matrix purity, cooling rate, sample holder design, optical alignment and excitation source (Hofstraat et al., 1989; Nakhimovsky et al., 1989). Some of these problems are fundamentally related to the non-equilibrium nature of the frozen solutions (Nakhimovsky et al., 1989), and are not easily solved for those compounds that are not fully compatible with the matrix. For most PAHs of the EPA priority pollutant list, however, reproducible Shpol'skii spectra can be obtained in n-octane matrices (Hofstraat et al., 1985), provided the sample holder and cooling regime is designed for instantaneous solidification (Hofstraat et al., 1989). When an appropriate internal standard is used, various experimental sources of error, such as solvent evaporation, the presence of air bubbles or cracks in the frozen sample, and variations in sample thickness, optical alignment or excitation energy, are adequately corrected for.

In the previous section, the quantitative determination of PAHs in standard solutions was described. In this section, we go one step further and test the applicability of the Shpol'skii method for PAH analysis in sediment reference materials from the National Research Council Canada (NRCC). The sediments were also analyzed by means of HPLC-fluorescence at the laboratory of the Tidal Waters Division of the Netherlands Ministry of Transport and Public Works, using the same extracts. The results are compared with the reference values provided by the NRCC.

EXPERIMENTAL

Origin of samples

Reference sediment materials HS-4, HS-6, and SES-1 were obtained from the NRCC. The HS-4 and HS-6 sediments originate from polluted harbors in Nova Scotia, Canada. The materials have been freeze dried, sieved over 125 μm mesh, and homogenized. PAH concentrations were determined by the NRCC Atlantic Research Laboratory in Halifax, Canada. Most PAHs are quantitated using five different methods: HPLC-absorption, HPLC-fluorescence, HPLC-MS, GC-MS, and GC-FID (Sim et al., 1988). The reference values in Tables I and II are based on 22-40 separate determinations; uncertainties represent 90 % confidence intervals.

SES-1 is a sediment sample from a pristine estuary, to which 15 PAHs have been added. In the ideal case, the spiking method would lead to a reference material in which the PAH content is known with almost absolute certainty, provided that mixing is complete, that losses are avoided, and that irreversible binding to particulate matter does not occur. However, actual concentrations, determined by the NRCC using the techniques mentioned above, were generally lower than the spike concentrations. Furthermore, systematic errors (biases) between the various analytical techniques were apparent; the average results reported by the NRCC with HPLC-fluorescence and GC-MS, the techniques yielding the highest and lowest values, respectively, for most compounds, are listed separately in Table III.

Extraction and liquid chromatography

Extraction and HPLC analysis of the samples was carried out at the laboratory of the Tidal Waters Division, using a standard procedure for the analysis of PAHs and PCBs in marine sediments and suspended matter. Ca. 2 g of dry sediment was Soxhlet extracted with acetone/hexane 1:3 (4 hours at 80 °C). After volume reduction, part of the extract was put aside for Shpol'skii analysis; part went through two further clean-up steps over Al_2O_3\Na_2SO_3 and SiO_2, respectively. HPLC separation was carried out using a HP 1090 chromatograph, equipped with a Vydac 201TP5 250 x 4.6 mm reversed-phase column (Mojave, Hesperia, CA). The following gradient elution programme was applied: water/methanol 30:70 at t = 0; rapid change to 80:20 at t = 5 min; gradual change to 100 % methanol at t = 50 min; return to start-up at t = 90 min. The flow rate was 0.9 ml/min. For fluorescence detection, the HP 1046A detector was

operated in 0th order mode: excitation wavelength 250 nm (band pass 12.5 nm); total emission above 300 nm was collected.

Shpol'skii spectrofluorimetry

The crude extracts, containing the PAHs at ca. 10^{-6} - 10^{-7} M concentrations, were diluted 1:1 with a 2 x 10^{-7} M solution of perdeuterated pyrene (internal standard) in n-octane. Hexane was carefully evaporated in a gentle stream of nitrogen and replaced with n-octane, a suitable matrix for most PAHs with 4-6 fused rings (Nakhimovsky et al., 1989). Samples were cooled to 26 K and analyzed using the lamp-excited Shpol'skii apparatus described in the previous section. For detection a Princeton Instruments IRY 1024 GRB intensified diode array was employed; the resolution was 0.10 nm. The emission of each compound was measured using the most optimal excitation wavelength, which is the resultant of the low-temperature absorption spectrum and the xenon lamp profile; the excitation wavelength should not be too close to the emission line of interest to prevent stray light problems.

Peak areas were divided by that of the internal standard and compared to previously determined calibration plots. In order to correct for matrix transmission losses in crude extracts (excitation light and emission from the analyte may be absorbed by the matrix, not necessarily to the same extent as for the internal standard), an absorption spectrum of the extract is recorded. The actual fluorescence intensity I, compared to the intensity I_n that would be observed in a transparent n-octane matrix, can be calculated from the matrix absorptions $A_{\lambda ex}$, $A_{\lambda em}$ at the excitation and emission wavelength of each compound:

$$\frac{I}{I_n} = \frac{1 - 10^{-(A_{\lambda ex} + A_{\lambda em})}}{(A_{\lambda ex} + A_{\lambda em}) \ln 10}$$

(front-face geometry; see also following section; Ariese et al., 1990). In practice, absorption of emission light was negligible; correction factors for absorption of excitation light were typically not larger than 10-20 % for the PAHs analyzed in these samples.

The HS-4 extract was also analyzed using a standard addition approach: a synthetic mixture, containing all PAHs to be determined at equal concentrations, was added to the sample at 9 x 10^{-8}, 3 x 10^{-7} and 9 x 10^{-7} M. The internal standard concentration was 1 x 10^{-7} M in all solutions. The analyte concentration in the original sample was calculated from the intercept, using simple linear regression.

RESULTS AND DISCUSSION

Shpol'skii analysis of reference material HS-4 was carried out using calibration graphs or a standard addition procedure. The results in Table I show excellent agreement between the two methods, indicating that the calibration curves, determined in clear standard solutions, are also applicable to crude sediment extracts. After correction for matrix absorption, the slope of the

calibration plots did not differ from that of the standard addition plots for the compounds listed in Table I. Since the standard addition method requires several measurements for each sample, the calibration graph method was preferred for the sediments HS-6 (Table II) and SES-1 (Table III).

Table I PAH concentrations (μg/g) in sediment reference material HS-4.

PAH[a]	HPLC-Flu[b]	Shpol'skii; calib.[c]	Shpol'skii; st. add.	NRCC values[d]
Pyr	0.69 ± 0.06	0.91 ± 0.04	0.88	0.94 ± 0.12
BaA	0.39 ± 0.02	0.44	0.46	0.53 ± 0.05
Chr	0.48 ± 0.09	0.38	0.39	0.65 ± 0.08
BaP	0.43 ± 0.04	0.53 ± 0.02	0.49	0.65 ± 0.08
BbF	0.48 ± 0.04	0.68	0.66	0.70 ± 0.15
BkF	0.28 ± 0.03	0.35 ± 0.03	0.33	0.36 ± 0.05
BgP	0.37 ± 0.06	0.40	0.39	0.58 ± 0.22

[a] Pyr = pyrene; BaA = benz[a]anthracene; Chr = chrysene; BaP = benzo[a]pyrene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; BgP = benzo[ghi]perylene; [b] results of 3 determinations; [c] results of 1-4 determinations; [d] uncertainties represent 90% confidence limits.

Table II PAH concentrations (μg/g) in sediment reference material HS-6.

PAH[a]	HPLC-Flu[b]	Shpol'skii; calib.[c]	NRCC values[d]
Pyr	1.98 ± 0.20	2.44 ± 0.17	3.0 ± 0.6
BaA	1.20 ± 0.11	NA	1.8 ± 0.3
Chr	1.50 ± 0.24	NA	2.0 ± 0.3
BaP	1.32 ± 0.22	1.69 ± 0.05	2.2 ± 0.4
BbF	1.81 ± 0.11	NA	2.8 ± 0.6
BkF	1.04 ± 0.13	1.01 ± 0.02	1.43 ± 0.15
BgP	1.48 ± 0.29	NA	1.78 ± 0.72

[a] See Table I for abbreviations; [b] results of 3 determinations; [c] results of 2 determinations; [d] uncertainties represent 90% confidence limits; NA = determination not attempted.

Table III PAH concentrations (μg/g) in sediment reference material SES-1.

PAH[a]	HPLC-Flu[b]	Shpol'skii; calib.[c]	NRCC values HPLC-Flu	NRCC values GC-MS
Pyr	1.58 ± 0.14	2.13 ± 0.17	3.5	2.4
BaA	0.29 ± 0.02	0.32 ± 0.04	0.8	0.5
Chr	0.85 ± 0.05	1.00	1.4	1.1
BaP	0.08 ± 0.01	0.08 ± 0.01	0.8	0.15
BbF	0.88 ± 0.05	1.6	1.2	[d]
BkF	0.76 ± 0.07	0.91 ± 0.08	1.0	[d]
BgP	0.69 ± 0.08	0.97 ± 0.13	0.8	0.69

[a] See Table I for abbreviations; [b] results of 3 determinations; [c] results of 1-3 determinations; [d] No GC data reported.

Comparison of the Shpol'skii results with the reference values for HS-4 and HS-6 indicate that, except for chrysene in HS-4, the data are within or just below the 90 % confidence interval stated by the NRCC. There seems to be a slight negative bias, which could be the result of incomplete extraction. The HPLC-fluorescence results, obtained at the Tidal Waters Division using the same extracts, also agree reasonably well with the reference values, although the negative bias is more pronounced. The accuracy of these HPLC-fluorescence measurements is much better than that reported by Sim et al. (1987), who stated that the poor resolving power of liquid chromatography combined with the relatively low selectivity of optical detection would preclude accurate measurements of PAHs in real samples.

For most PAHs analyzed in the SES-1 extract, agreement with the NRCC reference values (see Table III) is similar to that observed for the other sediment samples. Again, there seems to be an overall bias: for most compounds the average values increase in the order HPLC<Shpol'skii<NRCC. For benz[a]anthracene and benzo[a]pyrene, however, the HPLC and Shpol'skii results are close to each other but significantly lower than the NRCC values. Presumably this discrepancy between our results and the NRCC data cannot be caused by incomplete extraction, since the agreement is reasonably good for the other compounds in Table III. It is noted that the accuracy of the NRCC values is rather low: large biases are reported between the various analytical techniques used, especially for benzo[a]pyrene. The reported BaP concentrations in mg/kg are: 0.15 (GC-MS); 0.29 (HPLC-MS); 0.34 (GC-FID); 0.8 (HPLC-Flu.); 1.21 (spike level). According to the NRCC, the observed method biases cannot be explained by different extraction efficiencies. Other tentative explanations could be sample inhomogeneity or "ageing" (Landrum, 1989) of the sediment. Because of these unsolved problems, the reference material is, to our knowledge, no longer available.

Summarizing, we conclude that lamp-excited Shpol'skii spectrometry is a suitable technique for the quantitative analysis of a range of PAHs. Measurements could be carried out on crude Soxhlet extracts without further clean-up, thus reducing sample handling time and the risk of introducing contamination. Quantitative results were generally in good agreement with certified values, but large differences were found for some compounds in the SES-1 sample. We believe that the discrepancies observed do not originate from our analytical method. In fact, the SES-1 material seems not very suitable for reference purposes.

As a rule, "true" PAH concentrations in reference materials are determined by employing a spectrum of different analytical techniques. Since the selectivity of the Shpol'skii technique is based on spectral rather than physical separation, it is strongly recommended to include this fully independent method in quality control procedures.

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4.4

SHPOL'SKII FLUORIMETRIC DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN BIOTA

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ABSTRACT

The applicability of high-resolution Shpol'skii spectrofluorimetry to the direct analysis of polycyclic aromatic hydrocarbons (PAHs) in tern and mussel samples was investigated. The sensitivity of the measurements suffered considerably from the large amounts of interfering substances (e.g. fatty components) in the crude extracts, resulting in a poor-quality Shpol'skii matrix and a high sample absorbance. Nevertheless, after a thorough study of these limiting factors, optimal conditions could be defined and a number of PAHs were detected directly without any sample clean-up. Results obtained after sample pretreatment are also presented for comparison.

INTRODUCTION

The accurate determination of polycyclic aromatic hydrocarbon (PAH) pollution levels is important in environmental analytical chemistry. At several places along the Dutch coast the degree of pollution caused by these compounds, some of which are notorious carcinogens, is regularly monitored by the Ministry of Transport and Public Works. PAHs, being strongly apolar compounds, are hardly soluble in water, but rather adhere to organic material, and readily accumulate in the fatty tissues of living organisms. The direct determination of PAHs dissolved in sea water is very difficult owing to their extremely low concentrations (of the order of 10-100 pg/l for the Dutch coastal zone). As a result, tedious preconcentration techniques, which are prone to contamination effects, have to be employed in order to detect these sub-ng/l levels (Furuta and Otsuki, 1983). In another approach, the bioaccumulation of PAHs in living organisms is used to obtain measurable and time-integrated levels of the total biologically available fraction of these compounds in the area. The latter method is called active biological monitoring, and mussels have been demonstrated to be particularly useful for this purpose ("mussel watch"; Boom, 1987). A mussel constantly pumps and filters large amounts of sea water (ca. 50 l per day), and PAHs accumulate in the fatty tissues until the uptake is balanced by excretion and degradation and a plateau value is reached. Also, detection of PAHs in other biota is of interest for biologists and environmental chemists.

It is well known that some PAHs are highly carcinogenic, but carcinogenic activities may vary widely between closely related compounds (Hecht et al., 1976). For this reason, an analytical method for the determination of PAHs in environmental samples should not only be sensitive, but also highly selective. Until now the determination of PAHs in biota samples from the Dutch marine environment has routinely been carried out using reversed-phase liquid chromatography with fluorescence detection. The availability of an entirely different, independent technique, however, offers several advantages, like additional information or a possibility for intercomparison.

In this paper the application of high-resolution Shpol'skii spectrofluorimetry to the determination of PAHs in fatty samples of terns (*Sterna hirundo*) and mussels (*Mytilus edulis*) is reported for the first time. This technique, which enables one to obtain vibrationally resolved fluorescence spectra, is both highly sensitive and selective. Its usefulness has been demonstrated in environmental trace analysis, where even the structurally very similar methyl isomers of PAHs could be distinguished and separately determined (Garrigues and Ewald, 1985). Another advantage is that generally extensive clean-up is not needed; for instance, PAHs in sediment could be quantitatively determined directly in the extract (Hofstraat et al., 1985). The analysis of PAHs in biota is more difficult, owing to the low concentrations of the analytes and the high levels of potentially interfering substances. We report here how, using the Shpol'skii technique, a number of PAHs could be determined in a complex biotic extract without prior chromatographic separation or clean-up.

SHPOL'SKII FLUORIMETRY

Some fundamental aspects of the Shpol'skii method will be discussed very briefly here. Conventional fluorimetry is intrinsically a sensitive, but not a very selective, technique. Room-temperature fluorescence spectra of liquid solutions are generally broad and featureless. Consequently, fluorescence spectra cannot serve as a fingerprint for identification, and a mixture of fluorescent compounds can only be analysed in combination with a separation technique.

In the disordered liquid phase, spectral broadening can mainly be attributed to the fact that every solute molecule has different interactions with its solvent cage, resulting in different shifts of the energy levels. In addition, frequent collisions may cause a reduction of the average lifetime of the electronically and vibrationally excited states, which according to the Heisenberg principle leads to an increased uncertainty in the energy levels.

In the Shpol'skii approach these problems are tackled by measuring the sample in a suitable solvent (usually an n-alkane) at cryogenic temperatures. In the rigid, polycrystalline matrix formed, the solute will often have a certain favourable, well defined orientation within the crystal lattice. As all the individual guest molecules experience the same crystal field, their electronic transitions will all correspond to the same, well defined energies.

Since at the same time collisions are absent, line widths can be reduced to ca. 0.1 nm and a vibrationally resolved spectrum is obtained. The technique thus combines the selectivity of an infrared spectrum with the sensitivity of fluorimetry. More information about the basic principles and experimental details can be found elsewhere (Wehry, 1986; Hofstraat et al., 1988).

EXPERIMENTAL SECTION

After removing the shell, mussels were homogenized, freeze-dried, extracted with n-hexane in a Soxhlet apparatus and evaporated to dryness. Terns were plucked, freeze-dried, homogenized, freeze-dried again, extracted with hexane and evaporated to dryness. Of the fatty, brownish samples thus obtained, half was simply diluted with n-octane (Baker analyzed grade) and measured without further clean-up. The remaining part of the extracts was further cleaned over a silica gel column (15 g, deactivated with 5% water), and eluted with hexane. For the tern sample, this washing procedure had to be repeated several times in order to obtain a sufficiently clear extract. Before measurement a known concentration of internal standard was added, and hexane was replaced with octane through selective evaporation in a stream of nitrogen.

Samples were contained in a gold-plated copper sample holder, covered by a sapphire window, and mounted on the cold tip of a CTI Cryogenics Model 20 closed-cycle helium refrigerator (Waltham, MA). Four samples could be cooled simultaneously to 26 K. For excitation a 450-W xenon arc lamp and a Bausch & Lomb (Rochester, NY) monochromator, bandpass 8 nm full width at half maximum, FWHM), were used. For quantitative analysis, excitation and detection took place at the following wavelengths:

PAH	excitation (nm)	emission (nm)
Benz[a]anthracene	289	384.3
Benzo[a]pyrene	297	403.0
Benzo[b]fluoranthene	301	397.8
Benzo[ghi]perylene	302	419.6
Benzo[e])pyrene	292	388.3
Benzo[k]fluoranthene	308	403.6
Pyrene	335	372.0
Chrysene	268	360.6
Indeno[1,2,3-cd]pyrene	380	462.7
Perylene	391	444.7
Pyrene d_{10} (internal standard)	335	371.0

A 1-cm quartz cuvette with a 25% solution of nickel sulphate in water served as an extra band-pass filter, rejecting stray light very efficiently in the 370-440 nm region. Fluorescence from the samples was collected at 20° from the incident light by a positive lens, passed through a cut-off filter, and focused on the entrance slit of a Jobin-Yvon (Longjumeau, France) HR1000 monochromator, resolution 0.16 nm (FWHM). The signal was detected by a model 9558Q cooled photomultiplier tube (EMI, Middlesex, U.K.), operated at 1100 V, and processed by a laboratory-built preamplifier and a Stanford Research (Palo Alto, CA) SR400 photon counter. Spectra were recorded simultaneously by a Kipp & Zonen (Delft, The Netherlands) BD8 chart recorder and a Tulip XT ('s-Hertogenbosch, The Netherlands) IBM-compatible computer .

For quantitative determinations, perdeuterated pyrene was added as an internal standard to correct for variations in light intensity, optical alignment, irregularities in the sample, etc. Calibration graphs for octane as solvent for the intensity of the main emission line were straight from 10^{-6} M down to the detection limit. Detection limits varied from 3 x 10^{-10} M to 10^{-8} M, depending on the excitation efficiency, quantum yield, and relative intensity of the main emission peak. Absorption measurements were carried out on a Beckman (Fullerton, CA) DU-50 spectrophotometer using a 1-mm quartz cuvette.

RESULTS AND DISCUSSION

Crude samples

Attempts to obtain Shpol'skii spectra directly with the concentrated fatty extracts were unsuccessful. When the sample was diluted 1+9 with n-octane, on top of the broad emission a small narrow-banded signal was observed, which became stronger on further dilution. Maximal signal intensity was obtained for a dilution factor of about 100; further dilution resulted in a lower signal again (see Fig. 1a-d).

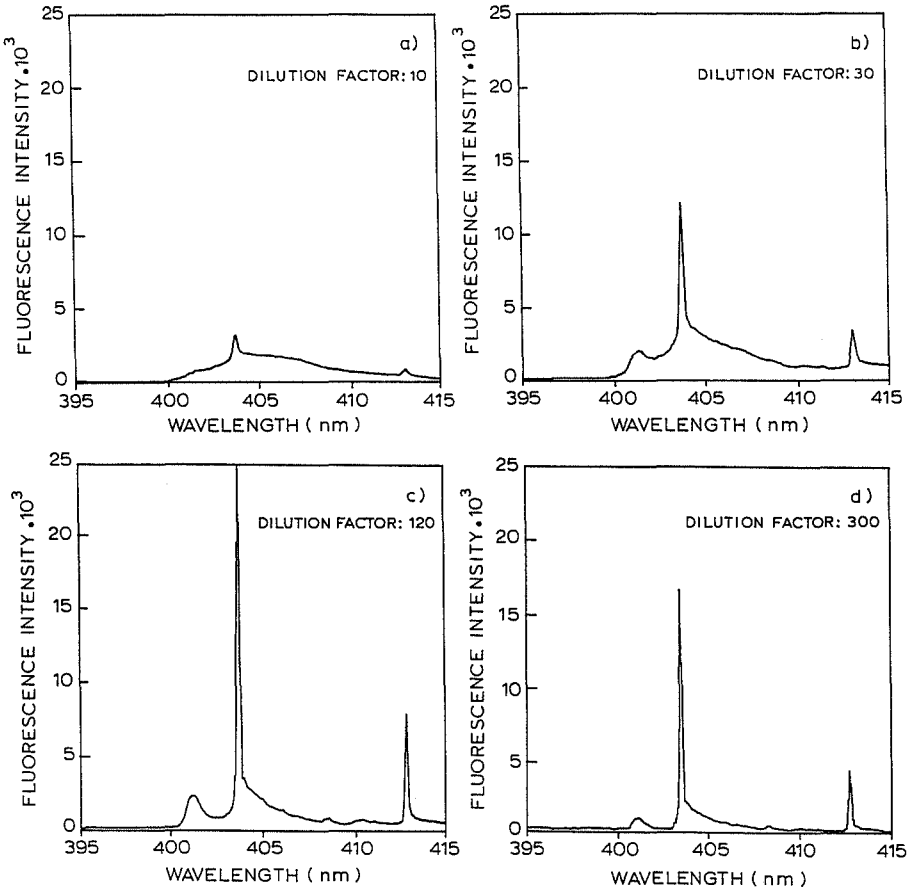

Fig. 1 Fluorescence spectra of the crude tern sample, spiked with benzo[k]fluoranthene, B(k)F, and successively diluted with octane; excitation, 308 nm; temperature, 26 K.
a) $f_d = 10$; [B(k)F] = 3 x 10^{-5} M. b) $f_d = 30$; [B(k)F] = 1 x 10^{-5} M. c) $f_d = 120$; [B(k)F] = 2.5 x 10^{-6} M. d) $f_d = 300$; [B(k)F] = 1 x 10^{-6} M.

The broad-banded emission cannot be due to freezing out of the analyte into amorphous intercrystalline regions as no differences were observed when various cooling rates were applied (Hofstraat et al, 1989). Nor can the signal reduction in concentrated samples be caused by reabsorption as vibronic bands are lowered to the same extent as the 0-0 emission line, and as the same effects were also observed for more diluted dilutions (10^{-7} M).

Two effects are to be discerned in figures 1a-d: on dilution there is a relative increase in the narrow-banded emission, and the total luminescence intensity fails to decrease linearly. These effects will now be explained separately.

To explain the first observation it should be realized that narrow-banded emission occurs only if at least a considerable percentage of the analyte molecules experiences more or less the same interaction with the surrounding matrix. This is known to be the general case for PAHs embedded in a regular alkane matrix, but guest molecules surrounded by one of the many possible mixed octane-fatty acid solvent cages will only contribute to a broad background signal. Assuming a random distribution of octane and fat, the statistical probability that a guest molecule happens to be surrounded exclusively by octane is given by:

$$P = (1 - \frac{1}{f_d})^n \qquad (1)$$

where f_d is the dilution factor, the term $(1 - 1/f_d)$ is the fraction (v/v) of octane after dilution and n is the number of octane molecules making up a regular Shpol'skii site. Figure 1 shows the fluorescence spectrum of the crude tern sample, spiked with benzo[k]fluoranthene and successively diluted with octane. The relative areas of the narrow Shpol'skii line and the broad background, compared to the areas obtained in neat octane solution, are as predicted by Eqn. 1. Assuming equal quantum yields and equally efficient excitation by the broad-banded light source,the experimentally observed relative intensity of the narrow Shpol'skii line is best fitted by a 'coordination number' n of about 35 (see Fig. 2). This seems a reasonable number as the guest molecule is expected to experience little interaction with matrix molecules outside the first solvation shell. Here, it may be tentatively mentioned that the smallest rectangular crystallite forming a substitutional site consists of 34 solvent molecules (4x3x3, with a medium-sized PAH taking the place of two octane molecules).

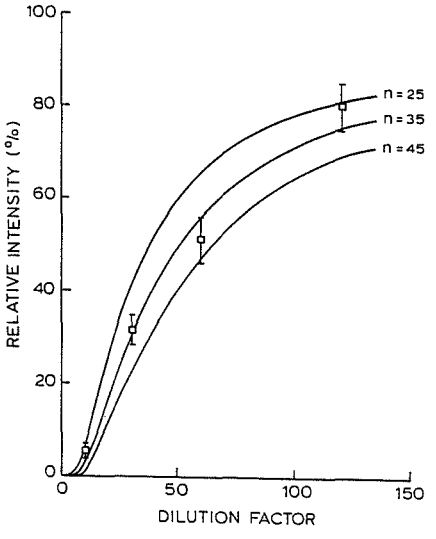

Fig. 2 Area of the main emission line relative to the total luminescence intensity between 400 and 410 nm, taking the intensity distribution in octane as 100 %. Solid lines: theoretical curves substituting various values of n into Eqn. 1

The second factor reducing the signal strength is the absorbance of the sample, owing to high concentrations of absorbing components in the fatty extracts. The room-temperature absorption spectrum of the yellowish tern sample (dilution factor 100) is shown in Fig. 3. If we assume the absorption spectrum of the matrix to be not very different at low temperatures, we can calculate the reduction of the fluorescence intensity due to absorption of both the excitation and the emission light in a front-face illumination geometry:

$$\frac{I}{I_n} = {}_0\int^D 10^{-A_{\lambda ex}(x)} * 10^{-A_{\lambda em}(x)}\, dx \qquad (2)$$

where I_n is the fluorescence intensity one would observe in neat octane solution and $A_{\lambda ex}$ (x) and $A_{\lambda em}$ (x) are the absorbances of the matrix at cell depth x at the excitation and emission wavelengths applied for a particular analyte. Owing to multiple scattering in the polycrystalline matrix, the effective path length D is roughly twice the actual cell depth (Soulignac and Lamotte, 1987), and is here set to 1.0 mm. Defining A_0 as the absorbance of the undiluted extract at cell thickness 1.0 mm (to be derived from Fig. 3) and integrating Eqn.2, the signal reduction in a diluted sample due to matrix absorption becomes:

$$\frac{I}{I_n} = \frac{f_d}{(A_{0,\lambda ex} + A_{0,\lambda em})\ln 10}\,[1 - 10^{-(A_{0,\lambda ex} + A_{0,\lambda em})/f_d}] \qquad (3)$$

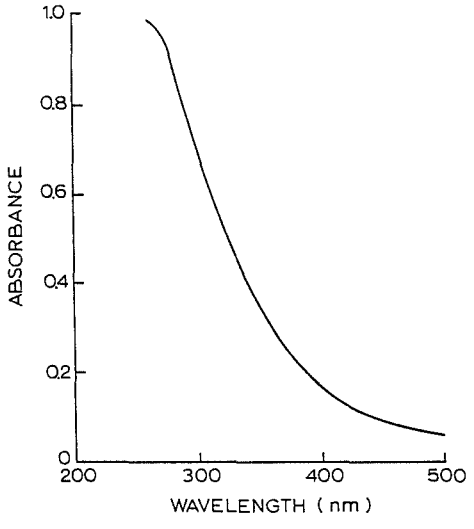

Fig. 3 Room temperature absorption spectrum of the crude tern sample, diluted 100 times with octane. Path length 1.00 mm.

A similar correction factor was derived by Leese and Wehry (1978). For benzo[k]fluoranthene, substituting $A_{0,308} = 64$ and $A_{0,404} = 16$ in Eqn. 3, the observed total luminescence intensities agree with the calculated matrix absorption within 10%.

Combining Eqns. 1 and 3, and including the effect of decreasing the analyte concentration, we obtain the following overall equation for the intensity of the narrow-banded emission of a particular analyte as a function of the dilution factor:

$$I(f_d) \sim \frac{c_0}{f_d}\left(1 - \frac{1}{f_d}\right)^n \frac{f_d}{(A_{0,\lambda ex} + A_{0,\lambda em})\ln 10}\left[1 - 10^{-(A_{0,\lambda ex} + A_{0,\lambda em}) / f_d}\right] \quad (4)$$

where c_0 is the PAH concentration in the undiluted extract. In this equation the first term decreases monotonously, while both the second and third terms increase monotonously to approach the asymptotic value of 1. The overall function shows an initial increase from zero, but then the signal response is almost constant over a large concentration range as the effect of a lower analyte concentration is approximately compensated for by the increasing regularity and transparency of the matrix. Of course, for more diluted samples the emission intensity decreases again. Figure 1 demonstrates these trends for benzo[k]fluoranthene and illustrates the flatness of Eqn. 4 over a wide range of f_d values: the dilution factor is in fact not a very critical parameter.

After this search for the optimum conditions, tern and mussel samples were diluted 100-fold with octane and measured at 26 K using pyrene d_{10} as internal standard. Although the background noise was not much higher than for neat academic solutions, the detection limits were of course greatly affected by the necessity of dilution. Nevertheless, we were still able to determine benzo[a]pyrene, benzo[k]fluoranthene and pyrene in both samples; the results are summarized in Table I, and Fig. 4a shows part of the tern emission spectrum, exhibiting the main fluorescence peaks of pyrene and the internal standard.

It should be realized of course that Eqn. 4 might have severe implications for quantitative measurements using an internal standard. Analytes of different sizes may have a different coordination number n. Further, the influence of matrix absorption may vary widely, depending on the excitation and emission wavelengths used. Therefore, a standard addition procedure was applied, spiking the tern sample with five PAHs (benz[a]anthracene, benzo[a]pyrene, pyrene, benzo[k]fluoranthene and perylene; still with pyrene d_{10} as internal standard; dilution factor 100). In general, the calibration graphs constructed for neat octane solutions proved to be applicable, which means that the signals from analyte and internal standard are both weakened to approximately the same extent. Table I shows that the results obtained via the internal standard and the standard addition methods agree, within experimental error. Only for perylene was a deviation of + 30% compared with the slope of the calibration graph found, which is indeed predicted considering the relatively high transparency of the matrix at the excitation and emission wavelengths used for this compound. For all other compounds checked, the deviation was less than 10%.

We conclude that, although quantitation using standard addition is of course basically more correct, for most compounds we may still use the calibration curves measured in pure octane, especially in strongly diluted solutions.

Table I Determination of PAHs in crude and neat samples.

PAH	tern before clean-up	tern after clean-up	mussel before clean-up	mussel after clean-up
Benz[a]anthracene		8		40
Benz[a])pyrene	9 (10)	4	27	17
Benzo[ghi]perylene		17		22
Benzo[k]fluoranthene	11 (11)	7	36	18
Chrysene		13		76
Pyrene	156 (153)	143	256	265
Perylene		2		8

Values are in ng/g organism; data in parentheses were measured using standard addition.

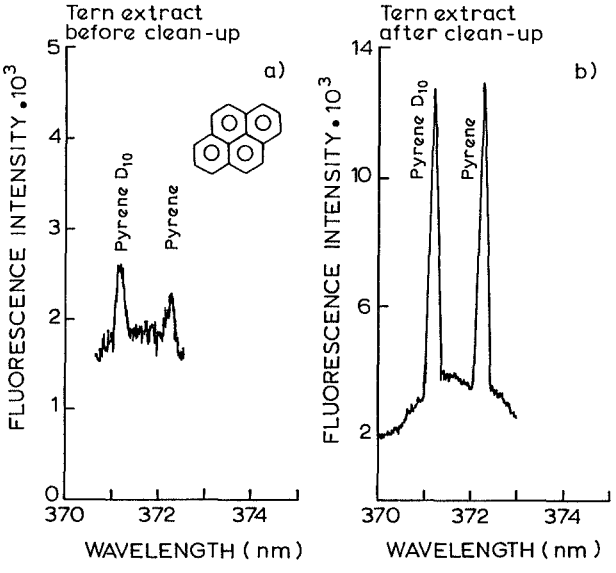

Fig. 4 Part of the Shpol'skii fluorescence spectra of the tern sample; excitation, 335 nm; temperature, 26 K. a) Before clean-up; [pyrene d_{10}] = 3 x 10^{-8} M; b) after clean-up; [pyrene d_{10}] = 1 x 10^{-7} M.

Neat samples

In a second series of experiments, an attempt was made to establish the extent to which the Shpol'skii analysis of biota samples would benefit from a sample clean-up. Using this procedure, the fatty components were largely removed and the samples became virtually transparent in the visible and near-UV regions. Strong dilution was no longer necessary, and much larger signals could be obtained in this way. This is illustrated by the spectrum for the cleaned tern sample in Fig. 4b, as compared to that of the crude sample in Fig. 4a. The samples were checked for ten different PAHs, of which seven could be determined (see Table 1). Benzo[b]fluoranthene, benzo[e]pyrene and indeno[1,2,3-cd]pyrene could not be detected. For pyrene, the standard deviation was found to be 4.7 % over seven independent measurements. For all compounds, the repeatability was better than 10%. In the original samples, the real PAH contents may have been higher than the values listed in Table 1, as the extraction may not have been complete.

The results obtained before and after sample clean-up indicate some loss of analytes as a result of the clean-up procedure. In a forthcoming investigation the extent of losses during the clean-up will be thoroughly examined. In fact, the Shpol'skii technique seems very useful as an independent method for determining recoveries of sample pretreatment steps, as it offers the unique possibility to perform direct analysis both in complex matrices and in clean solvents.

This appears to be the first report of the application of Shpol'skii spectrofluorimetry to the direct determination of PAHs in fatty biotic samples. Fatty acids and other kinds of non-polar compounds hardly interfere with the measurements, as long as their total content is not much larger than 1 % after dilution with octane. Of course, the dilution step leads to a severe decrease in sensitivity, but still three different aromatic compounds could be detected at the ng/ml level in the crude extract.

We believe that Shpol'skii spectrofluorimetry is a reliable and generally applicable method for the determination of PAHs in complex samples. The technique will prove to be particularly useful when unambiguous identification at trace level is required, but some form of sample pretreatment will often be necessary to reach the required sensitivity.

The detection potential is expected to be considerably improved if a laser is used for excitation instead of a xenon arc lamp. The laser not only provides higher light intensities, but can also be more selective owing to its high monochromaticity (the S_1-S_0 Shpol'skii absorption spectrum is also narrow-banded). Application of laser-excited Shpol'skii spectroscopy (LESS) (D'Silva and Fassel, 1984) to the determination of PAHs in biotic samples is currently under study.

ACKNOWLEDGEMENTS

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4.5

SHPOL'SKII FLUORIMETRIC DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN COMPLEX ENVIRONMENTAL SAMPLES

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ABSTRACT

High-resolution fluorimetry in low-temperature n-alkane Shpol'skii matrices is a powerful technique for the analysis of rigid, non-polar compounds like polycyclic aromatic hydrocarbons. Because of the method's sensitivity and selectivity, sample clean-up, preconcentration, and even chromatographic separation can often be left out. The Shpol'skii analysis of pyrene in crude extracts from marine sediments and from bird meat is demonstrated. Special attention is focussed on the extra possibilities acquired when a laser is used as excitation source (laser excited Shpol'skii spectroscopy, LESS).

INTRODUCTION

For the analysis of polycyclic aromatic hydrocarbons (PAHs) one can often make good use of their strong native fluorescence. Unfortunately, room-temperature fluorescence spectra of liquid solutions are usually quite congested and contain not enough information for identification purposes. For the same reason, complex mixtures of fluorescent compounds can only be analyzed after chromatographic separation.

One way to obtain high-resolution fluorescence spectra is to cool the aromatic compounds in a suitable solvent (usually an n-alkane) to cryogenic temperatures (Shpol'skii et al., 1952). Upon freezing, the analytes are trapped in a polycrystalline matrix, in which they occupy specific sites. This way the inhomogeneity of the solvent is strongly reduced, as, ideally, every analyte molecule will have the same, well-defined orientation within the crystal lattice. Additionally, freezing eliminates collision-induced homogeneous broadening as well as dynamic quenching processes.

In Fig. 1 the Shpol'skii effect is illustrated for a pyrene solution in n-octane. Cooling results in a remarkable gain in spectral resolution (line width ca. 0.1 nm), while at the same time sensitivity is increased by two orders of magnitude. The vibrationally resolved spectrum can be used for fingerprint identification, and since the spectra from different compounds hardly suffer from spectral overlap, it is often possible to analyse a mixture of PAHs without prior separation (Hofstraat et al., 1985).

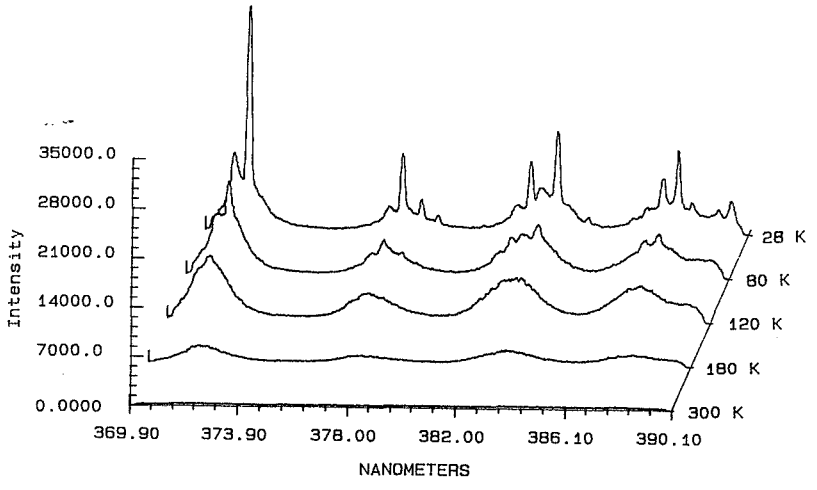

Fig. 1 Temperature dependence of pyrene emission in n-octane; conc. = 1.0 x 10^{-6} M, lamp exc. 335 nm.

In this short communication the analytical potential of the Shpol'skii technique is illustrated for pyrene in environmental matrices, but obviously the method is equally suitable for the analysis of other PAHs in complex samples. It will be shown how the use of a laser excitation source leads to a remarkable increase in sensitivity and selectivity.

EXPERIMENTAL

Sample preparation

A sediment sample from the Uithuizerwad (Wadden Sea, The Netherlands) was homogenized, freeze-dried, and Soxhlet extracted with hexane/acetone 3:1 during 4 hours. After addition of an internal standard (pyrene d_{10}) the solvent was replaced with n-octane and the crude extract was cooled and analyzed without further clean-up.

A tern (*Sterna hirundo*) was plucked, homogenized, freeze-dried and Soxhlet extracted in the same manner. After addition of the internal standard and solvent replacement, the crude extract had to be diluted with n-octane due to the high content of extractable lipids. To obtain a good homogeneous Shpol'skii matrix the alkane content should be at least 98-99 %. (section 4.4; Ariese et al., 1990).

Apparatus

Samples were contained in a gold-plated copper sample holder, covered by a sapphire window and mounted to the cold station of a CTI Cryogenics model 20 closed-cycle helium refrigerator (Waltham, MA). Four samples could be cooled simultaneously to 26 K. For lamp excitation we used a 450 W xenon arc lamp and a Bausch & Lomb monochromator (Rochester, NY; band pass 8 nm). A 1 cm cuvette with a 25% $NiSO_4$ solution served as an extra band pass filter. For LESS measurements, a frequency-doubled Quantel Nd-YAG laser was used in combination with a Rhodamine 6G dye laser (Quantel) and an angle-tuned mixing crystal, yielding a tunable spectral range from 365-375 nm. Emission was collected at a 20° angle from the incident light by a positive lens and dispersed by a Jobin-Yvon HR1000 monochromator (Longjumeau, France; resolution 0.16 nm) or a Spex 1877 triple monochromator (Edison, NJ; resolution 0.07 nm). The light was detected by a cooled photomultiplier tube (RCA 31034-2A; Lancaster, UK) and a Stanford Research SR400 photon counter (Palo Alto, CA), or by an intensified diode-array detector (Princeton Instruments IRY 1024 GRB). For time-resolved measurements, the latter could be gated with a Princeton Instruments FG100 pulse generator.

RESULTS AND DISCUSSION

Conventional, lamp excited Shpol'skii spectroscopy

Sediment samples Fig. 2 shows part of the Shpol'skii emission spectrum from the crude sediment extract in n-octane. The excitation wavelength (335 nm) is optimal for pyrene, but with broadband lamp excitation other compounds may be excited, too. The spectrum contains also emission lines from the internal standard pyrene d_{10} , as well as some minor contributions from other analytes. The discrete emission lines not only allow for a definite identification, but can also be used for direct quantitation (Hofstraat et al., 1985).

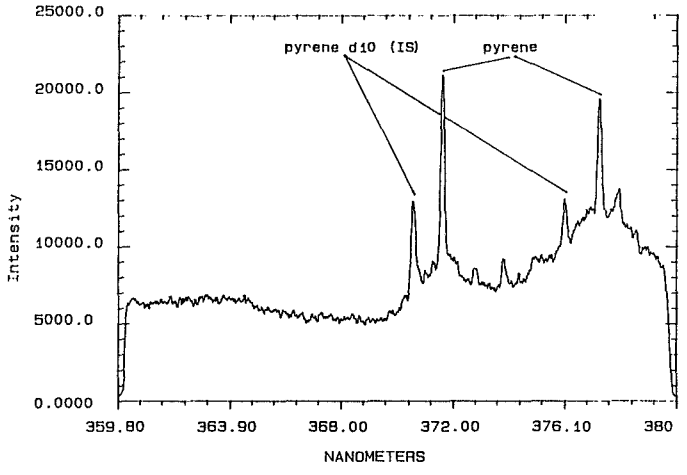

Fig. 2 Emission from sediment extract in n-octane at 28 K; int. standard = 1.33 x 10^{-7} M, lamp excitation 335 nm.

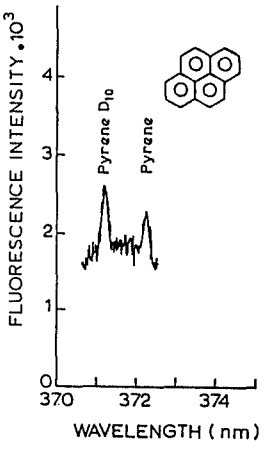

Fig. 3 Emission from crude conc. tern extract in n-octane at 26 K; conc. int. standard 3.0 x 10^{-8} M; lamp excitation 335 nm.

Biotic samples The analysis of PAHs in biotic matter is usually more difficult, owing to the lower PAH concentrations and the high contents of extractable lipids that may interfere with the measurements. In order to obtain a regular, and relatively transparent Shpol'skii matrix, the crude extract must be sufficiently diluted with octane, resulting, of course, in a loss of sensitivity. Still we were able to record an acceptable Shpol'skii spectrum: Fig. 3 shows the main emission lines from pyrene and from the internal standard in a crude extract of a tern. If better sensitivity is required, one can perform a simple chromatographic clean-up over SiO_2 ; once the lipids are removed from the extract, dilution is no longer necessary and stronger signals are obtained (section 4.4; Ariese et al., 1990).

Laser Excited Shpol'skii Spectroscopy

The use of a laser excitation source may offer a number of important advantages for the analysis of PAHs in complex environmental samples (D'Silva and Fassel, 1984).

High excitation power Compared to the light intensity of a xenon arc lamp dispersed by a high-throughput monochromator, a Nd-YAG/dye laser combination may offer an increase in excitation power of typically two orders of magnitude. Furthermore, the laser beam can easily be focussed on a very small (microliter) sample volume. This is important, as in high-resolution spectroscopy the fluorescent spot has to be projected onto the very narrow entrance slit of the emission monochromator. Overall, we can obtain a total increase in the effective excitation power of three orders of magnitude.

High monochromaticity Since the S_1-S_0 region of the Shpol'skii absorption spectrum is also narrow-banded (Nakhimovsky et al., 1989), it is possible to selectively excite one particular compound in a mixture. Fig. 4 shows a 3-dimensional excitation-emission spectrum of pyrene in n-octane. Emission spectra were continuously recorded as the laser was tuned through the 0-0 transition. Pyrene d_{10} was also present in the mixture, but although its excitation wavelength is shifted over only one nanometer, it is completely invisible, as the laser line does not match exactly. It is clear that we have here a powerful tool to increase the emission of a particular analyte and at the same time reduce interferences from other compounds. Fig. 5a shows the pyrene emission from the crude tern extract using laser excitation.

Time-resolved detection If the laser system is of the pulsed type, we can use time-resolved detection to discriminate between the relatively long-living (20-500 ns) emission of the aromatic analytes and instantaneous processes like stray-light or (Raman) scattering. Also short-

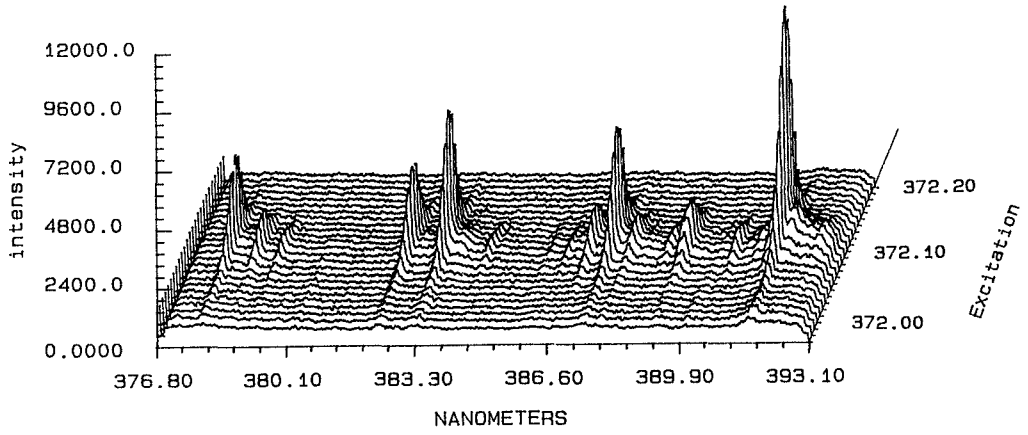

Fig. 4 Excitation-emission plot of pyrene in n-octane at 28 K; conc. = 5.0 x 10^{-7} M, laser excitation.

living background luminescence is removed. A pulse generator was used to activate the photocathode of the diode-array detector 50 ns after the laser shot. The effect of time-resolution is illustrated if we compare the signal-to-noise ratios in Figs. 5a and b. Of course, for shorter-living analytes a shorter delay must be used and the background noise may not be removed completely.

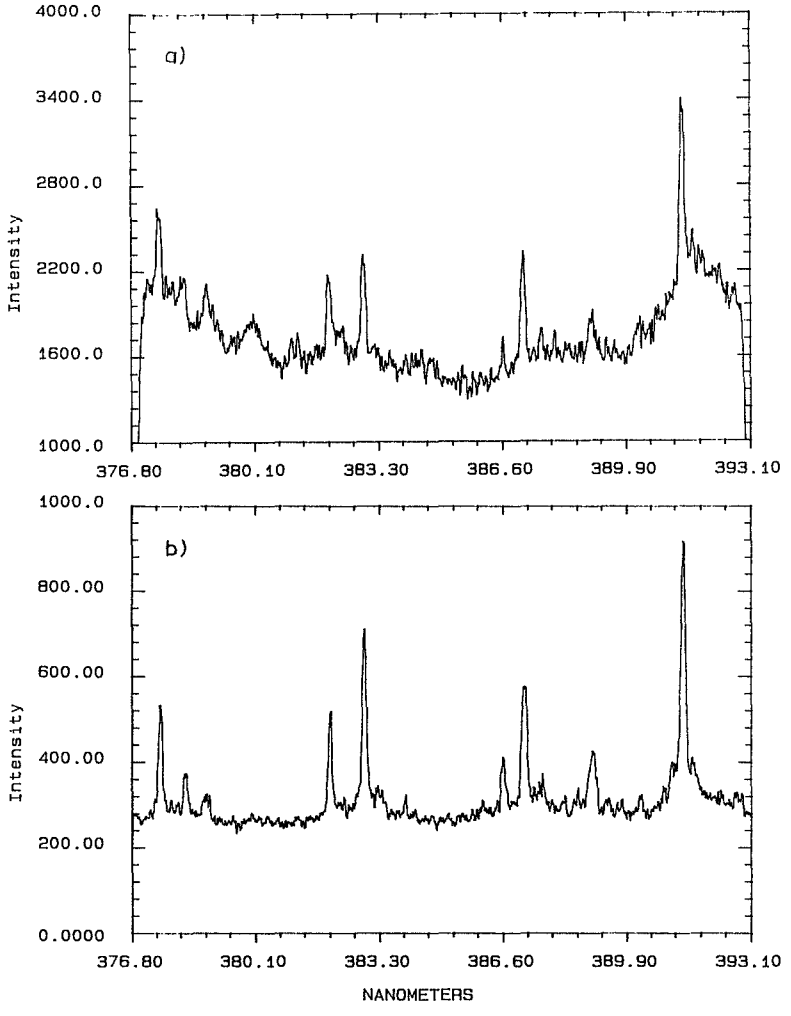

Fig. 5 Shpol'skii emission of pyrene in crude tern extract in n-octane at 28 K; laser excitation 372.10 nm. a) no time resolution; b) with time-resolved detection (delay = 50 ns).

The overall spectral improvement due to laser excitation is clearly demonstrated if we compare Fig. 5b to Fig. 3, showing the pyrene emission from the same crude tern extract. The advantages are obvious: both sensitivity as well as selectivity are remarkably improved.

FUTURE PROSPECTS

It has been demonstrated previously that the Shpol'skii technique can be used to qualitatively and quantitatively determine PAHs in environmental samples (Hofstraat et al, 1985; Garrigues and Ewald, 1985). The technique is very sensitive: if an optimal photomultiplier tube is used, the limit of detection for pyrene, in an synthetic mixture of 10 PAHs in n-octane using conventional lamp excitation, is as low as 5×10^{-10} M. For benzo[a]pyrene the detection limit is an order of magnitude lower; with laser excitation even two orders of magnitude (for a 10 µl sample volume this means 50 attomoles. This high sensitivity is especially important if only very small amounts of sample are available or if preconcentration methods are suspected to cause contamination effects.

The selectivity of the method can be an important advantage if, for instance, isomers have to be distinguished. It is well known that the methyl substitution pattern strongly influences the carcinogenic activity of isomeric alkylated PAHs (Conney, 1982).

Few compounds show native fluorescence, and only rigid compounds of low polarity will show narrow-banded fluorescence under Shpol'skii conditions. This implies on the one hand that the technique is applicable to only a limited number of compounds, but on the other hand it means that no or only a very simple sample clean-up is required. Since every chemical manipulation may bring about random and/or systematic errors, regarding analytical accuracy direct methods are to be preferred. Finally, it is noted that the use of Shpol'skii spectroscopy as an independent reference technique may contribute to the reliability of analytical data (section 4.1; Mastenbroek et al., 1990).

For trace level analysis of PAHs in complex samples, it will be essential to employ laser excitation. Of course, one should realise that laser systems offering acceptable powers in the near-UV region are still quite expensive, and an extensive wavelength range can not be reached without having to change the dye solution or the system configuration. Nevertheless, we are convinced that for a number of complicated analytical problems (laser excited) Shpol'skii spectroscopy can offer an adequate solution.

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CHAPTER 5

SHPOL'SKII SPECTROFLUORIMETRIC ANALYSIS OF PAH METABOLITES

5

CHEMICAL DERIVATIZATION AND SHPOL'SKII SPECTROFLUORIMETRIC DETERMINATION OF BENZO[a]PYRENE METABOLITES IN FISH BILE

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ABSTRACT

The practical applicability and analytical performance of laser-excited Shpol'skii spectroscopy (LESS) for the determination of benzo[a]pyrene (BaP) metabolites was studied. Quantitation of these compounds in fish bile can provide insight into the BaP uptake from a contaminated aquatic environment (biomonitoring). Chemical derivatization of hydroxylated BaP metabolites with methyl iodide (to improve the compatibility with the Shpol'skii matrix) was tested. The methylation of phenolic metabolites was rapid and quantitative, but with BaP-dihydro diol metabolites, mixtures of methylation and elimination products were formed. An analytical procedure was developed for the quantitative determination of 3-OH BaP, the major BaP metabolite detected in bile from flounder (*Platichthys flesus*). With LESS, the detection limit was 0.005 ng/ml (200 attomole); the repeatability was 16 %. The method was applied to a mesocosm experiment in which flounders were exposed to different sediments. The average BaP uptake from Rotterdam harbor sediment was 40 times higher than the uptake from Wadden Sea sand. Direct contact with the sediments was a major route of exposure. The usefulness of the method for the biomonitoring of polycyclic aromatic hydrocarbon stress in the aquatic environment is discussed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are considered an important class of environmental pollutants. PAHs may originate from incomplete combustion or petrochemical contamination, and are relatively persistent in the environment. Many PAHs show mutagenic and/or carcinogenic activity (Pelkonen and Nebert, 1982), and may constitute a threat to human health. PAH pollution can also affect animal life, especially in the aquatic environment (Mix, 1986; Couch and Harshbarger, 1985). High incidences of liver tumors in fish at particular sites were found to be correlated to high levels of PAHs in the sediments (Malins et al., 1988; Baumann et al., 1987).

In order to monitor exposure to xenobiotics in the environment, the determination of their concentrations in the various environmental compartments may not be sufficient, since bioavailability is not taken into account. Biological monitoring, that is, the determination of a particular compound (or its metabolites) in a specific organism or tissue, can provide valuable information on the actual uptake rate (Landrum, 1989) . Fish usually do not show considerable accumulation of PAHs (Varanasi et al., 1985). Upon absorption, PAHs are rapidly metabolized into more polar derivatives that are stored in the gall bladder to be excreted (Varanasi et al., 1986; Goddard et al., 1987). Attempts to biomonitor PAH uptake by fish should therefore concentrate on PAH metabolites in excreta rather than on parent PAHs in tissue. Krahn and coworkers (1984) developed HPLC/fluorescence and GC-MS methods to determine PAH metabolites in fish bile.

The potent carcinogen benzo[a]pyrene (BaP) is often used as a model compound to study the toxic effects of PAHs (Phillips, 1983). BaP is metabolized by hepatic enzyme systems into a number of mono- and polyhydroxylated derivatives. Some reactive species may form adducts with proteins or DNA, but most metabolites are rapidly excreted in the form of glucuronide, sulfate, or glutathion conjugates (Leaver et al., 1992). In laboratory experiments, the biotransformation products of BaP are usually analyzed by means of HPLC; to avoid problems with detection sensitivity, toxicologists can administer high doses of BaP or use radioactive material. In the field, the concentration of BaP metabolites in bile of feral fish could be used as an indicator of exposure to BaP and related PAHs in the area, but the detection of BaP metabolites requires extremely sensitive and selective methods. Bile of fish exposed to many different PAHs will contain an even more complex mixture of PAH metabolites that may interfere with the analysis. Using HPLC/fluorescence, Krahn and colleagues (1987) succeeded in detecting 3-hydroxy BaP in some bile samples from a highly polluted site near Seattle, but sub-ppb sensitivity would be needed for detection of BaP exposure in other, less polluted areas. As an extra complication, the bile volumes available are usually not sufficient for trace enrichment.

Shpol'skii spectrometry may offer the required sensitivity and selectivity. The method is based on the fact that some compounds, when cooled in a suitable crystalline matrix to cryogenic temperatures, will show highly resolved fluorescence spectra (Shpol'skii, 1962; Nakhimovsky et

al., 1989). The technique combines fingerprint identification with excellent sensitivity and allows the determination of complex mixtures without chromatographic separation (Morel et al., 1991; Hofstraat et al., 1985). The analytical performance can be further improved when a tunable laser is used as excitation source (laser-excited Shpol'skii spectrometry, LESS) (D'Silva and Fassel, 1984; section 4.5; Ariese et al., 1991). Shpol'skii spectra have been published for a large number of compounds, mainly non-polar, planar, rigid molecules like PAHs that can substitute one or more solvent molecules in an n-alkane crystal (Nakhimovsky et al., 1989). In a few cases, the Shpol'skii effect was observed for more polar compounds, like PAH metabolites (Khesina et al., 1975; Garrigues and Ewald, 1985). Weeks and coworkers (1990) described a procedure to transform mono-hydroxy benz[a]anthracenes into less polar methoxy derivatives, which could subsequently be analyzed by means of LESS. Recently, the same research group reported the derivatization and Shpol'skii spectra of a wide range of BaP metabolites: mono-hydroxy BaP derivatives, BaP dihydro diols, BaP dihydro diol epoxide, as well as BaP tetrahydro tetrol (Weeks et al., 1991).

In this paper we investigate the practical applicability of the Shpol'skii technique to the analysis of BaP metabolites in fish bile. The derivatization reaction was critically studied for phenolic and dihydro diol metabolites of BaP. An analytical method was developed for the quantitation of 3-OH BaP in bile samples. The model fish studied was the flatfish species flounder (*Platichthys flesus*). Exposure to BaP was realized via parenteral injection or in mesocosm systems containing different sediments. Some field samples were also analyzed.

EXPERIMENTAL SECTION

Chemicals and safety

All 12 mono-hydroxy BaP isomers, BaP-*trans*-9,10-dihydro diol, BaP-*trans*-7,8-dihydro diol, BaP-*cis*-4,5-dihydro diol and BaP-*trans*-4,5-dihydro diol, were obtained from the NCI Chemical Carcinogen Repositories (MRI, Kansas City). Benzo[a]pyrene was purchased from Radiant Dyes (Wermelskirchen, Germany). Perylene d_{12} was purchased from Merck Sharp & Dohme (Montreal, Canada). β-glucuronidase 30 U/ml with arylsulfatase activity 20 U/ml was supplied by Merck. n-Octane was obtained from Janssen Chimica, all other solvents were Baker analyzed grade. The chemicals were used without further purification. Since BaP is a potent carcinogen, protective gloves should be worn when handling BaP solutions or during dissection of BaP-injected fish. Most BaP metabolites are considered non-toxic, but care should be taken when handling 2-hydroxy BaP, 11-hydroxy BaP or BaP-*trans*-7,8-dihydro diol (Pelkonen and Nebert, 1982). As the derivatization reaction is carried out with strong base and with the very toxic methyl iodide, the use of a fume cupboard and of protective clothing is highly recommended.

Fish exposure studies

We studied flounder bile samples representing a wide range of BaP exposure levels. Highest exposure levels were realized by administering a single dose of BaP (parenteral injection in acetone/Mulgofen 620; 0.78 or 4.04 mg/kg bodyweight). The fish were fed shrimp (*Crangon crangon*) until two days before injection and were sacrificed 48 hours after injection. To simulate semi-chronic exposure to realistic BaP pollution levels, flounders were kept during four weeks in three different mesocosms: 1) moderately polluted Rotterdam harbor sediment (dredging class II, direct contact with the sediment was possible); 2) Indirect exposure to Rotterdam harbor sediment (Wadden Sea sand bottom; food and water equilibrated with the polluted harbor sediment; 3) Wadden Sea sand bottom (control group). The PAH contents of the sediments (fine fraction only) were determined after wet sieving over a 63 μm nylon filter, by means of HPLC with fluorescence detection (Klamer et al., 1990). The harbor sediment contained 450 ppb BaP and 800 ppb pyrene; the Wadden Sea sand contained 140 ppb BaP and 180 ppb pyrene (ng/g dry weight of fine fraction). The fish were fed until two days before section to allow the accumulation of metabolites in the gall bladder and to reduce the confounding effects from different feeding habits. Finally, field samples were analyzed from flounders captured at the Wadden Sea and at a more remote part of the North Sea (53°44'N; 6°30'E). Bile was collected from the gall bladder by means of a syringe and stored in vials in the dark at -20 °C until further use.

Sample treatment

20 μl of bile was diluted with water to 1 ml and incubated during 2 hours at 37 °C with 20 μl of β-glucuronidase/arylsulfatase solution to hydrolyze conjugated metabolites. Typically, maximum yield was reached within 20-30 minutes. The free metabolites were quantitatively extracted by repeated extractions with n-hexane (4 times 3 ml). We observed that for the extraction of deconjugated PAH metabolites from bile samples, the use of solid phase C18 cartridges often resulted in lower extraction efficiencies, less effective clean-up and irreproducible yields. The latter was presumably caused by the varying concentrations of surfactants in the samples. For direct analysis of underivatized metabolites, hexane was evaporated in a stream of nitrogen and the residue dissolved in 2 ml of n-octane. In most cases, however, the volume of the extract was reduced to ca. 0.5 ml and the metabolites derivatized according to a procedure adopted from Weeks and coworkers (1990): 2 mg of sodium hydride was washed three times with n-pentane in a flask under nitrogen atmosphere. 1 ml of dimethyl sulfoxide (DMSO) was added, the mixture was stirred at 70 °C for several minutes until the formation of H_2 bubbles had ceased. Subsequently, the bile extract was added; the reaction mixture was vigorously stirred for another 10 minutes at 60 °C, then cooled to room temperature. Finally, 100 μl of methyl iodide was added; after several minutes of stirring the reaction was quenched with 4 ml of water. The methylated products were quantitatively extracted with 2 times 3 ml of n-hexane. For Shpol'skii analysis, the extract was concentrated and the solvent gradually replaced with n-octane in a gentle stream of nitrogen. The exact end volume was determined by weighing (ca. 400 μl). n-Hexane

was preferred as extraction solvent over n-octane because of its lower boiling point and higher purity. For quantitation, perdeuterated perylene was added to the final analytical sample as an internal standard; 2 x 10^{-8} M for bile samples from the most polluted mesocosm, 2 x 10^{-9} M for the other samples. When a number of derivatizations had to be carried out, a slightly different procedure was followed: a larger volume of methyl iodide solution in basic DMSO was prepared, and divided over the bile extracts in disposable glass vials at room temperature. This time-saving method also eliminates the risk of memory effects. For the methylation of phenolic BaP derivatives, both methods yielded equal results (cf. below).

Shpol'skii spectrometry

For selective laser-excitation a Quantel frequency-doubled Nd:YAG laser was used to pump an oxazine 170 dye laser; frequency mixing with the fundamental 1064 nm output yielded a tunable range from 409 to 419 nm. The beam intensity was typically 1 mW per 3 mm^2 cross-section; repetition rate 10 Hz. In case short-wavelength, non-selective excitation was needed, the dye laser output was frequency doubled to yield 348 nm radiation. In some cases, broad-banded xenon lamp excitation at 300 nm was employed. Four samples (ca. 10 µl) could be cooled simultaneously to 23 K by a CTI Cryogenics (Waltham, MA) closed-cycle helium refrigerator. The spectral resolution did not improve significantly when the samples were cooled to 10 K. Front-face illumination was applied; fluorescence emission was collected at a 20 ^{o} angle, dispersed by a Spex 1877 triple monochromator (1200 grooves/mm grating; resolution 0.1 nm) and detected by an intensified diode-array detector from Princeton Instruments (type IRY 1024 GRB). For gated detection the Princeton Instruments GF-100 pulser unit was used.

RESULTS AND DISCUSSION

Direct Shpol'skii analysis of BaP metabolites

First, we explored the practical applicability of the Shpol'skii technique to the direct analysis of BaP metabolites (without chemical derivatization). Quasilinear spectra of some mono-hydroxy metabolites have been reported for 3-OH BaP and 6-OH BaP (Khesina et al., 1975), and for 9-OH BaP (Garrigues and Ewald, 1985), but no mention was made of applications to real analytical problems.

As expected, using a gold-plated copper sample holder with sapphire windows designed for optimal thermal conductivity and instantaneous solidification of small (10 µl) sample volumes, the phenolic metabolites 1-OH BaP, 3-OH BaP and 9-OH BaP all yielded narrow-banded spectra in n-octane at 23 K. Nevertheless, there is strong evidence that these analytes are not fully compatible with the crystalline n-octane host: 1) Some broad-banded background fluorescence was always observed; the relative intensity would increase when applying a less optimal cooling regime. 2) Spectra of 9-OH BaP at different concentrations showed different site distributions. 3) Despite the good fluorescence quantum yields at room temperature (Khesina et

al., 1975), detection limits were unexpectedly high (at best 10^{-8} M for 3-OH BaP, using Xe lamp excitation at 300 nm, more than an order of magnitude worse than for the parent compound BaP under similar circumstances). 4) At high concentrations (10^{-5} M), no relative decrease of the 0-0 emission line due to reabsorption was observed. These findings suggest that, as the result of the polar phenolic group, most analyte molecules freeze out of the crystalline matrix during the cooling procedure and give rise to a broad background or form non-fluorescent aggregates (Hofstraat et al., 1989). Consequently, the actual concentration of isolated analyte molecules that produce quasilinear emission is considerably lower than the analytical concentration. Apart from the obvious loss of sensitivity, the fact that the shape and intensity of the Shpol'skii spectra depend critically on concentration, cooling rate or the presence of polar impurities in the matrix, will make proper quantitation in real samples very difficult (Rima et al., 1982).

1-OH BaP and 3-OH BaP (but not 9-OH BaP) were detected in bile of flounder after injection with BaP (Ariese et al., 1993a), but one should not forget that the average daily intake of BaP by fish in a polluted environment will be several orders of magnitude lower than the applied dose. For reasons of sensitivity, and also repeatability, we concluded that Shpol'skii spectrometry is not a suitable method for the direct determination of mono-hydroxy BaP metabolites in field samples, and that derivatization would be required.

Evidently, we did not obtain analytically useful narrow-banded spectra for the still more polar dihydro diol metabolites (BaP-*trans* -9,10-dihydro diol, BaP-*trans*-7,8-dihydro diol, BaP-*cis*-4,5-dihydro diol and BaP-*trans*-4,5-dihydro diol). Attempts to use more polar solvents or mixed solvents as a Shpol'skii host for these metabolites were unsuccessful.

Derivatization of phenolic metabolites

In order to render the analytes more compatible with the n-octane matrix, the derivatization procedure described by Weeks and coworkers (1990) was adopted. Testing the reaction for 1-OH BaP, 3-OH BaP and 9-OH BaP, we found that the methylation of phenolic BaP derivatives with methyl iodide in basic DMSO is indeed a very rapid reaction: at high analyte concentration, the formation and methylation of a BaP phenolate is visualized by instantaneous color changes. Since the derivatization reagents do not interfere with the analysis, they can be added at large excess. In that case, the kinetics of the reaction are of the pseudo-first order type and the derivatization is equally fast at low analyte concentrations. Quenching the reaction after 10 seconds or after 10 minutes resulted in equal yields. Analyzing the reaction mixtures with thin layer chromatography, HPLC, and Shpol'skii spectrometry, we never detected any unreacted starting material or side products. In contrast with the derivatization of dihydro diol metabolites (cf. below), the methylation of phenols was found to be a practical, straightforward and quantitative reaction, irrespective of reaction time or temperature (20 or 60 °C).

The 12 mono-hydroxy derivatives of BaP were methylated and their Shpol'skii spectra recorded using non-selective laser excitation at 348 nm. In n-octane, the 12 methoxy derivatives all produced different fingerprint spectra, making isomer-specific determination possible (Fig. 1)

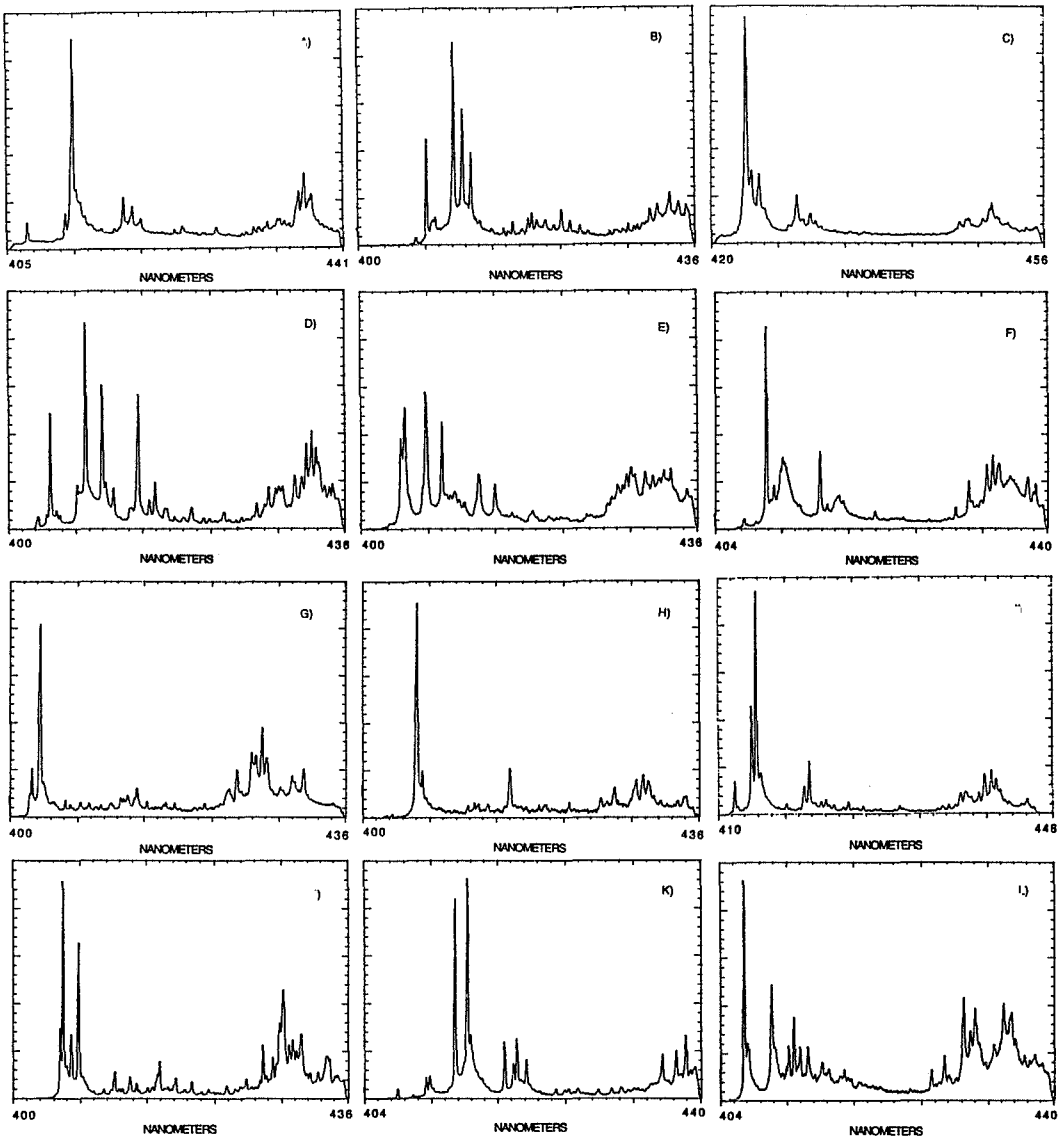

Fig. 1 Shpol'skii spectra in n-octane of phenolic BaP metabolites after derivatization, using non-selective laser excitation at 348 nm. A) 1-methoxy BaP, 10^{-7} M; B) 2-methoxy BaP, 10^{-6} M; C) 3-methoxy BaP, 2 x 10^{-7} M; D) 4-methoxy BaP, 10^{-6} M; E) 5-methoxy BaP, 10^{-6} M; F) 6-methoxy BaP, 10^{-6} M; G) 7-methoxy BaP, 10^{-6} M; H) 8-methoxy BaP, 10^{-6} M; I) 9-methoxy BaP, 10^{-6} M; J) 10-methoxy BaP, 10^{-6} M; K) 11-methoxy BaP, 10^{-6} M; L) 12-methoxy BaP, 10^{-6} M.

In case of 3-OH BaP, the improved host-guest compatibility after derivatization resulted in a 20-fold increase in quasilinear fluorescence intensity; the detection limit improved to 5.0 x 10^{-10} M (lamp excitation at 300 nm). Furthermore, the spectra were fully reproducible, irrespective of the cooling procedure, and concentration-dependent site distributions were not observed for any of the mono-methoxy derivatives. In conclusion, the derivatization of phenolic metabolites prior to Shpol'skii analysis results in a considerable improvement of both sensitivity and reproducibility.

Derivatization of dihydrodiol metabolites

The bioactivation of BaP to mutagenic intermediates occurs primarily through mono-oxygenation at the 7,8 or at the 9,10 position. The resulting epoxides are usually hydrolyzed to the respective dihydro diols, which may either be metabolized further or conjugated and excreted (Gelboin, 1980). The development of a sensitive, isomer-specific technique for the analysis of dihydro diol metabolites in biological matrices would be of great importance. Weeks and colleagues (1991) presented LESS spectra of the derivatization products of BaP-*trans*-7,8-dihydro diol and BaP-*trans*-9,10-dihydro diol. Apparently, the methylation of vicinal, aliphatic hydroxy groups would proceed in the same manner as in the case of phenolic compounds, and no mention was made of the formation of side products. Since, however, previous attempts in our laboratory to derivatize BaP dihydro diols always led to more than one product, we decided to study the reaction in more detail. When the reaction was carried out at 60 °C, as outlined in the experimental section, the derivatization product of BaP-*trans*-9,10 dihydro diol showed room temperature fluorescence excitation- and emission spectra characteristic for mono-hydroxy BaP derivatives, but very different from that of the starting material, indicating that the chromophore had undergone an important change. GC-MS analysis revealed the presence of two compounds with identical mass spectra: molecular ion 282; major fragments 267 (M - CH_3) and 239 (M - CH_3OC), analogous to the fragmentation of methoxy naphthalene (Heller and Milne, 1978). Even when direct-inlet/chemical ionization mass spectroscopy was applied to the product mixture, no compound with molecular ion m/z = 314 was detected. Finally, the reaction products were fractionated by means of preparative normal-phase HPLC (Lichrosorb Si-60 5 µm packing; mobile phase 100 % n-hexane), and unambiguously identified as 9-methoxy BaP and 10-methoxy BaP. Their respective Shpol'skii spectra were identical to those presented in Fig. 1(I) and 1(J). Apparently, at elevated temperatures and in prolonged contact with a strong base, BaP-*trans*-9,10-dihydro diol undergoes elimination of water, yielding one of two possible phenolates which are subsequently methylated as soon as CH_3I is added to the reaction mixture. The driving force of the reaction is, of course, the restoration of the full aromaticity of BaP. In a similar way, the methylation of BaP-*trans*-7,8-dihydro diol yielded a mixture of 7-methoxy BaP and 8-methoxy BaP when carried out at elevated temperatures. Methylation of BaP-*trans*-4,5-dihydro diol led to a mixture of 4-methoxy BaP, 5-methoxy BaP and a third, unidentified compound. The mono-methoxy isomers were identified using the Shpol'skii spectra in Fig. 1.

In an attempt to suppress the elimination reaction, the basic DMSO solution was first cooled to 20 °C, and the time lapse between the addition of BaP-*trans*-9,10-dihydro diol and methyl iodide was minimized. Best results were obtained when CH_3I was added prior to the metabolite. The room temperature methylation of BaP-*trans*-9,10-dihydro diol yielded a main product with fluorescence excitation- and emission spectra similar to that of the starting reagent. Using thin layer chromatography (RP-18 plates from Merck, mobile phase acetonitrile/water 90:10 v/v), a new product was detected, as well as at least one minor side product with the retention factor of 9-methoxy BaP. We found no evidence of unreacted starting material. The direct-inlet mass spectrum of the product mixture showed the presence of a compound with m/z = 314, with major fragments 283 (M - CH_3O) and 252 (M - 2 x CH_3O), which is in full agreement with the expected dimethylated product. The spectrum also contained the masses 282, 267 and 239, characteristic for mono-methoxy BaP (elimination products). Using preparative normal-phase HPLC (mobile phase: hexane/isopropanol 95:5 v/v), the main product was separated from the side products 9-methoxy BaP and 10-methoxy BaP and its Shpol'skii spectrum in n-octane solution could be recorded (Fig. 2).

All evidence suggests that the major product of the room temperature derivatization of BaP-*trans*-9,10-dihydro diol was indeed the desired BaP-*trans*-9,10-dihydro dimethoxy. The Shpol'skii spectrum shown in figure 2, however, bears no resemblance to that presented by Weeks et al. (1991), which cannot be explained by differences in excitation wavelength or cooling rate, but rather indicates that the derivatization reaction may also lead to other products than the three compounds identified in this study. Unfortunately, even at room temperature, the formation of elimination products could not be entirely avoided.

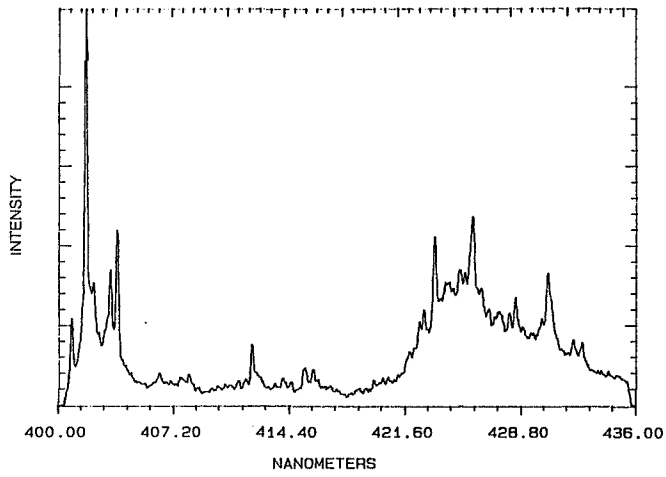

Fig. 2: Shpol'skii spectrum of purified main product in n-octane after room-temperature derivatization of BaP-*trans*-9,10-dihydro diol. Conc. = 1 x 10^{-7} M; λ_{exc} = 348 nm.

We conclude that great care has to be taken when using the derivatization reaction described above for the analysis of BaP dihydro diols: the formation of the corresponding dihydro dimethoxy compound may not be quantitative, while, on the other hand, the detection of 4- and 5-methoxy BaP, 7- and 8-methoxy BaP, or 9- and 10-methoxy BaP does not necessarily indicate the presence of the corresponding phenolic metabolites in the original sample. Similar problems were encountered by Jacob et al. (1989): upon treatment of pyrene 4,5 dihydro diol in rat urine with strong acid (prior to methylation with diazomethane and GC analysis), large amounts of 4-OH pyrene were formed. An extra complication for quantitative applications is the fact that the appearance of the Shpol'skii spectrum (site distribution and relative intensity of the broad-banded background) was found to be dependent on concentration and cooling rate. The same phenomenon has been observed for other, not fully compatible host-guest combinations, e.g. acenaphthene in n-hexane (Hofstraat et al., 1989).

Determination of BaP metabolites in fish bile

In a first explorative study, bile samples were analyzed from fish that had received a high dose of BaP via injection. Before hydrolysis, no fluorescent metabolites could be extracted with hexane, indicating a high degree of conjugation (Leaver et al., 1992). A typical Shpol'skii spectrum of a bile extract after enzymatic hydrolysis and methylation at 60 °C is shown in Fig. 3. Non-selective laser excitation at 348 nm was employed, in order to be able to determine all metabolites simultaneously; the overall dilution factor was 1000. The spectrum is dominated by 3-methoxy BaP and 1-methoxy BaP (compare with reference spectra 1(C) and 1(A)); the relative contribution of the latter varied considerably between individuals: between 7 and 26 % of the total amount of metabolites detected.

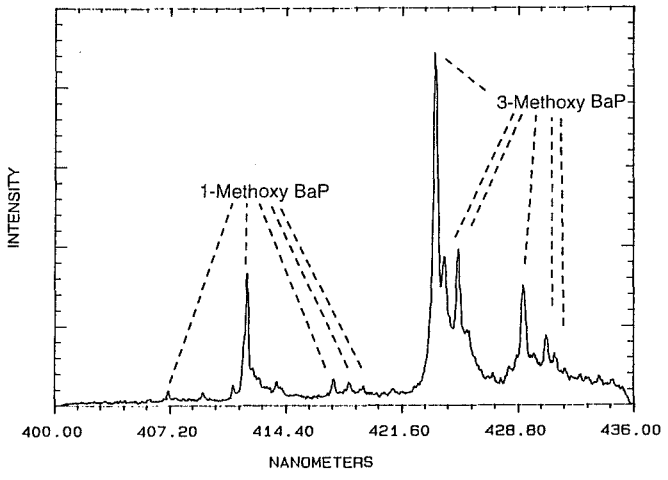

Fig. 3 Shpol'skii spectrum of methylated flounder bile sample (0.78 mg/kg BaP injected), featuring 1-methoxy BaP and 3-methoxy BaP. λ_{exc} = 348 nm.

In some samples, using longer detector exposure times, traces of 7- and 8-methoxy BaP, or of 9- and 10-methoxy BaP could be detected. These compounds were not detected (7-, 8-, or 10-methoxy BaP) or present at much lower level (9-methoxy BaP) when the same bile samples were derivatized at room temperature, indicating that they were elimination products from their respective diols. The amount of 3-methoxy BaP and 1-methoxy BaP determined did not depend on derivatization conditions. Dimethyl derivatives of BaP dihydro diol metabolites were not observed directly, which can be explained by the fact that their detectability in Shpol'skii matrices is one or two orders of magnitude worse than that of mono-methoxy derivatives, as was also noticed by Weeks and coworkers (1991).

The fact that 3-OH BaP and 1-OH BaP are major metabolites in fish bile is in full agreement with the measurements of Krahn et al (1987). Within 48 hours following parenteral injection, generally more than 50 % of the administered dose had accumulated in the gallbladder as (conjugated) 3-OH BaP. At present, we have no explanation for the observation that BaP-*trans*-9,10-dihydro diol and BaP-*trans*-7,8-dihydro diol contribute only marginally (< 1 %) to the (hydrolyzable) BaP metabolite profile in flounder bile.

Because of the fact that: 1) BaP is predominantly excreted in the bile of flounder as 3-OH BaP, 2) the derivatization of 3-OH BaP is a fast and quantitative reaction, 3) 3-methoxy BaP yields good and reproducible Shpol'skii spectra, it was decided to develop a procedure for the quantitative determination of trace levels of 3-OH BaP in fish bile and to use the latter as a marker compound for the biomonitoring of BaP uptake from a polluted environment.

Quantitative determination of 3-OH BaP

In the field, fish are exposed to a complex mixture of xenobiotics, including PAHs. Since each compound may be biotransformed into several different metabolites, the composition of excreta (e.g. bile fluid) is likely to be even more complex. For the spectrofluorimetric determination of a specific analyte in an extract containing a multitude of fluorescent compounds, it is generally advantageous to choose the excitation- and emission wavelengths close to each other, and so reduce the number of potential spectral interferences. Furthermore, in a Shpol'skii matrix, only the S_1-S_0 part of the excitation spectrum consists of narrow lines (Nakhimovsky et al., 1989), and is suited for selective excitation with a narrow-banded light source. The optimal excitation wavelength for 3-methoxy BaP was found to be 418.36 nm, only 5.2 nm apart from the 0-0 transition, the most intense emission line. Stray light was very effectively rejected by a triple monochromator. The remaining sources of background noise in spectra of solvent blanks were detector thermal noise, detector read-out noise, Raman lines and quasilinear emission from impurities (e.g. perylene). In some cases, time-resolved detection was applied to reduce thermal noise or to distinguish between Raman lines and quasilinear fluorescence. The main emission line of 3-methoxy BaP, however, did not suffer from spectral overlap. In practice, gating was found to decrease the absolute sensitivity of our intensified diode array detector and turned out to be of limited value for the analysis of real samples. The limiting factor in bile extracts was usually

broad background fluorescence, and the average fluorescence lifetime of the background was not significantly different from that of the analyte (ca. 10 ns).

For quantitation, an internal standard must be added to the analytical sample to compensate for variations in sample thickness, laser power, and optical alignment. In order to assure proper correction for laser output fluctuations (meanwhile saving time as well), the internal standard should be excitable at the wavelength chosen for the analyte, and should have a sufficiently strong emission line in the emission window covered by the multichannel detector. Perdeuterated perylene was found to meet the above requirements; ratioing the peak areas of the 0-0 emission lines, a straight calibration curve was obtained for 3-methoxy BaP in the concentration range of interest (3×10^{-11} M - 1×10^{-8} M). The perylene d_{12} concentration was kept constant at 2×10^{-9} M. Photochemical decomposition of the analyte was observed, but was less than 5 % during the usual irradiation time of 120 s. To assure correct quantitation, the calibration solutions were irradiated during the same period.

The absolute detection limit (S/N = 3) for 3-methoxy BaP, using laser excitation at 418.36 nm, was found to be 5×10^{-12} M (50 attomole) in n-octane solutions. The detection in bile extracts was not seriously affected by matrix interferences: when the sample treatment was carried out without overall dilution (provided that sufficient bile was collected), the detection limit was still 2×10^{-11} M or 0.005 ng/ml. For most samples, we used an overall dilution factor of 20; in that case the detection limit was 2×10^{-10} M or 0.05 ng/ml in the original sample, which was sufficient to detect exposure to BaP in all samples from the mesocosm experiment. The repeatability of the method (4 replicates of sample extraction and determination) was 16 %. Every day, a standard solution was analyzed to check for any deviations from the calibration curve.Because of the selectivity of excitation, a small change in laser wavelength would lead to gross analytical errors, but that was never observed.

Biomonitoring of BaP exposure

Laser excited Shpol'skii spectrometry of 3-OH BaP was applied to a mesocosm study in which flounders were exposed during four weeks to three degrees of pollution as described in the experimental section. Mesocosm 1 and 3 reflect the range of PAH pollution levels encountered in the Dutch coastal waters and estuaries. Mesocosm 2 was designed to find out what route of exposure contributes most significantly to the total BaP uptake. Hydrolyzed bile samples were derivatized at room temperature. After addition of the internal standard, the extracts were cooled to 23 K and their Shpol'skii spectra recorded using selective laser excitation at 418.36 nm. For the mesocosm samples, short-wavelength excitation at 348 excitation could not be used because of two reasons: limited sensitivity and spectral overlap with emission bands from 1-methoxy pyrene (1-OH pyrene is usually present at much higher levels in fish bile, see Table I). Notwithstanding the 20-fold dilution caused by the sample workup, 3-methoxy BaP could be detected in all samples, even from mesocosm 3 (Fig. 4). The multiplet structure from Fig. 1(C) has disappeared as the result of site-selective excitation. The analytical results, summarized in

Table I, show that fish exposed to Rotterdam harbor sediment had absorbed and metabolized 40 times more BaP than fish from the Wadden Sea sand basin. Furthermore, fish from the second mesocosm showed only a 6-fold increase, indicating that some uptake of BaP can take place through the water phase or through the diet (Malins et al., 1985), but that direct contact with the sediment is the major route of exposure for a bottom-dwelling fish like flounder. Direct absorption through skin or gills, or ingestion of PAH-containing particles, may both be important factors.

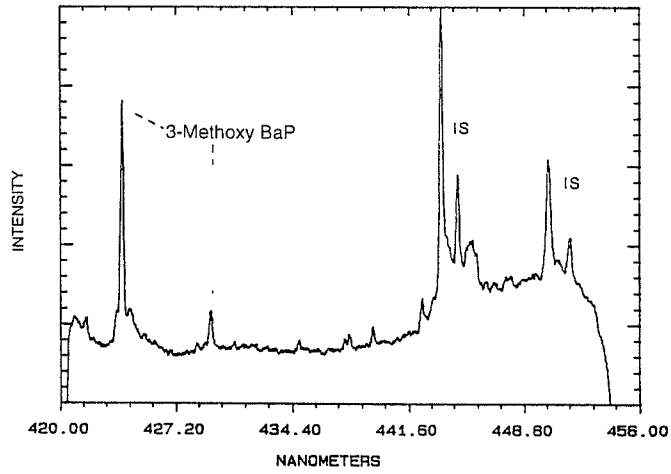

Fig. 4 Shpol'skii spectrum of methylated bile extract from Wadden Sea mesocosm, featuring 3-methoxy BaP. Site-selective laser excitation at 418.36 nm; IS = perylene d_{12}.

Table I also lists 1-hydroxy pyrene levels determined by means of synchronous fluorescence spectrometry (SFS) (section 6.1; Ariese et al., 1993b). Although 1-methoxy pyrene can be measured with LESS in an n-octane matrix (Ariese et al., 1993c), the relatively high concentrations allowed the determination (in a large number of samples) with a faster, more conventional method. The 1-OH pyrene data show a similar trend as the results for 3-OH BaP, but the 1-OH pyrene concentrations are a factor of 300-600 higher, which is not explained by the relative contents of the parent PAHs in the sediments (see Experimental Section), nor by the fact that 3-OH BaP is not the only metabolite of BaP. Apparently, the bioavailability of pyrene is much higher than that of BaP, which agrees with the kinetic studies of Landrum (1989). The standard deviations reported in Table I are an indication of the usual biological spread (Varanasi et al., 1986).

Table I Pyrene- and BaP metabolites in fish bile after exposure to different mesocosms.

	3-OH BaP (ng/ml)	1-OH pyrene (ng/ml)
1) HARBOR SEDIMENT (direct contact possible)	50 ± 36 (n=9)	15900 ± 6700 (n=23)
2) HARBOR SEDIMENT INDIRECT (PAH uptake through food and/or water)	7.7± 2.4 (n=4)	2600 ± 1500 (n=16)
3) SAND BOTTOM	1.2± 0.1 (n=3)	800 ± 480 (n=26)

Data are expressed as the arithmetic mean ± standard deviation (number of samples).

Finally, a limited number of field samples was analyzed: a pooled bile sample from flounders from the Wadden Sea was found to contain 1.6 ng/ml 3-OH BaP, comparable to the levels encountered in the samples from mesocosm 3 (Wadden Sea sand bottom). Considerably lower exposure to BaP takes place at open sea: two bile samples from the North Sea contained levels of 3-OH BaP just above the detection limit (ca. 0.005 ng/ml).

CONCLUSIONS

Direct Shpol'skii analysis of underivatized mono-hydroxy BaP metabolites in fish bile is possible, but has its drawbacks. The technique could be used in laboratory studies if unambiguous isomer-specific identification of biotransformation products is required. These analytes, however, are not fully compatible with the low-temperature n-octane host. Consequently, the spectra depend rather critically on experimental conditions, and the sensitivity is insufficient for most field applications. A rapid and quantitative derivatization reaction with methyl iodide in basic DMSO yields methoxy derivatives that give intense, high-quality Shpol'skii spectra in n-octane matrices, and which are very suitable for trace determination with LESS.

The derivatization of BaP dihydro diol metabolites was found to lead to a mixture of the dimethylated analyte and elimination products; the relative yields depend critically on reaction conditions. Even when alternative, straightforward reaction schemes will be found that yield quantitative results, the detectability of the non-planar dihydro dimethoxy compounds in a Shpol'skii matrix is not nearly as good as that of the mono-methoxy BaP derivatives. The isomer-specificity of the method could be useful for the study of BaP metabolism pathways, but the limited sensitivity is expected to hamper most applications.

BaP administered to flounders through parenteral injection was mainly excreted as conjugated 3-OH BaP; 1-OH BaP was the second major metabolite. Traces of 9-OH BaP, BaP-*trans*-9,10-dihydro diol and BaP-*trans*-7,8-dihydro diol were identified in some samples.

An important finding is that, within 48 hours following parenteral injection, generally more than 50 % of the administered dose had accumulated in the gallbladder as (conjugated) 3-OH BaP. This means that, even without induction, the mixed-function oxygenase (MFO) activity in flounder liver is rather high, and certainly not a rate limiting factor if fish are exposed to sub-microgram amounts of BaP in the field. Thus, the 3-OH BaP concentration in bile is a straightforward indicator of the amount of BaP absorbed, not confounded by differences in metabolic activity. For the biomonitoring of BaP exposure in the field, it is an additional advantage that the metabolite concentration in bile reflects only the most recent uptake. It is, therefore, a good indicator of BaP stress in the area of capture.

An analytical procedure was developed for the quantitative determination of 3-OH BaP in fish bile. Employing enzymatic hydrolysis, chemical derivatization, and laser excited Shpol'skii spectrometry, the detection limit is as low as 0.005 ng/ml, which is amply sufficient for the biomonitoring of BaP uptake in the Dutch coastal waters. Some extra effort will be required to monitor the much lower levels of BaP pollution at open sea. Application of the technique to a mesocosm experiment revealed that exposure to PAH-contaminated harbor sediment leads to a strongly increased uptake of BaP as compared to the reference group. Direct contact with the sediment or ingestion of particles are major uptake routes.

The 1-hydroxy pyrene levels in bile correlate well with the 3-OH BaP data, but the absolute amounts of pyrene absorbed and metabolized are more than two orders of magnitude larger than that of BaP. On the other hand, the pyrene content of the sediments was not even twice as high as that of BaP: the PAH uptake profile is clearly very different from the PAH profile in the sediment. This illustrates the value of a biomonitoring approach: the actual integrated exposure of an organism, which is the resultant of environmental levels and the bioavailability of the compound via various uptake routes, is measured directly. When PAH metabolite determination in bile of bottom-dwelling fish gains more widespread acceptance as a useful tool for the biomonitoring of PAH exposure in the aquatic environment, then fast and cheap analytical methods that can be applied to large numbers of samples will undoubtedly be preferred. In that case, determination of 1-OH pyrene with synchronous scanning fluorimetry (section 6.1; Ariese et al., 1993b), or measurement of "total bile fluorescence" with HPLC (Krahn et al., 1986) would be very useful as a simple screening method. That approach seems justified, since comparison of our data in Table I with the results of Krahn and coworkers (1987) indicates that the PAH metabolite profile in bile of flatfish appears to be quite constant. For a limited number of samples, one could use a more sensitive and specific technique like laser-excited Shpol'skii spectrometry to quantitate the relatively small, but toxicologically relevant uptake of potent carcinogens like BaP.

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CHAPTER 6

SYNCHRONOUS FLUORESCENCE SPECTROMETRY OF PAH METABOLITES

6.1

SYNCHRONOUS FLUORESCENCE SPECTROMETRY OF FISH BILE: A RAPID SCREENING METHOD FOR THE BIOMONITORING OF PAH EXPOSURE

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ABSTRACT

The uptake of polycyclic aromatic hydrocarbons (PAHs) by fish can be determined by screening the gall bladder bile for PAH metabolites. Conjugated 1-hydroxy pyrene is a major metabolite in bile of fish exposed to PAH polluted sediment. Synchronous fluorescence spectrometry is presented as a rapid screening technique for the determination of this compound; the complete analysis takes only 2-3 minutes. HPLC with fluorescence detection was used to validate the assay. Calibration methods, using either free 1-hydroxy pyrene or the pyrene-1-glucuronide conjugate as a reference standard, were investigated. The technique was applied to a mesocosm study in which the uptake of PAHs by flounder (*Platichthys flesus*) from polluted sediment was studied. Direct contact with the sediment proved the most important factor; the uptake through water phase or diet was less significant. The usefulness of 1-hydroxy pyrene analysis in bile as a monitoring tool for PAH exposure is discussed.

INTRODUCTION

Environmental pollution with polycyclic aromatic hydrocarbons (PAHs) continues to be a matter of great concern. PAHs in the aquatic environment originate mainly from incomplete combustion processes and from petrochemical pollution (National Research Council, 1985). PAHs are primarily bound to sediment particles, but may still be biologically available to marine organisms (Landrum, 1989; Varanasi et al., 1985). Upon uptake by fish, some PAHs, e.g. benzo[a]pyrene (BaP), can be metabolized into reactive intermediates that form adducts with DNA; the biochemical mechanisms are similar to those observed in mammals (Varanasi et al., 1986; Von Hofe and Puffer, 1986). Although there is no definite proof for a causal connection, the high incidence of liver neoplasia in fish at particular locations is believed to be related to PAH pollution (Malins et al., 1988; Mix, 1986; Baumann et al., 1987).

Routine monitoring of PAH levels in the aquatic environment usually involves the determination of parent PAHs in sediment samples. Additional information on the actual uptake of these compounds by aquatic organisms can be obtained via a biomonitoring approach. Generally, the biotransformation rates of PAHs in fish are fairly high, which means that accumulation is not likely to occur, and analysis of parent PAHs in liver or muscle tissue will reveal only very low levels (Varanasi et al., 1985; Dunn, 1991). Therefore, the biomonitoring of PAH uptake should concentrate on the determination of PAH metabolites in excreta, in particular in gall bladder bile (Krahn et al., 1984; Maccubbin et al., 1988). Bile analysis has also proved very suitable to study the uptake of other xenobiotics, like saturated hydrocarbons (Hellou and Payne, 1987), or chlorinated organics (Oikari and Kunnamo-Ojala, 1987).

The bile of PAH-exposed fish contains a multitude of oxygenated PAH derivatives, usually in the form of glucuronide, sulfate, or glutathion conjugates. Analytical protocols for the determination of PAH metabolites in fish bile have been developed by Krahn and coworkers (1987). The analysis involves an enzymatic treatment to hydrolyze most conjugates, the free metabolites are subsequently extracted and quantitated by means of high performance liquid chromatography (HPLC) with fluorescence detection, or gas chromatography (smaller metabolites only). However, a complete analysis of all detectable PAH metabolites would be rather difficult (complexity of the sample, lack of standards), costly, and time-consuming. If one is merely interested in monitoring the relative PAH stress at a particular location, it would be advantageous to introduce a single parameter that is indicative of the overall PAH uptake. Krahn and coworkers have chosen to standardize the reversed phase HPLC separation and measure the integrated fluorescence intensity detected at 380/430 nm, the optimal excitation/emission wavelengths for 3-hydroxy BaP. The resulting quantity is often expressed in BaP equivalents, although one should be aware that the largest contribution to the total fluorescence stems from metabolites of smaller PAHs (e.g. pyrene, fluoranthene), that are absorbed, metabolized, and excreted in far greater amounts than BaP (Krahn et al., 1987). The "total bile fluorescence" approach has been successfully applied to a number of ecotoxicological studies in the field as

well as in the laboratory (Johnston and Baumann, 1989; Collier and Varanasi, 1991). However, there are two drawbacks to the method. Firstly, although the procedure is not overly complicated, the total analysis time (hydrolysis, extraction, and HPLC separation) is still considerable. As a result of the typical biological variability encountered in bile samples, large numbers of samples will have to be analyzed to obtain statistically acceptable data, and a more rapid technique would be welcome. Secondly, "total bile fluorescence" is essentially an arbitrary quantity, which cannot be validated with an independent technique. The dependence on sample treatment (hydrolysis and clean-up) and instrumental parameters (especially the spectral band pass of the fluorescence detector, which has a strong influence on the response factor of compounds that do not have a maximum of absorption/emission at the selected wavelengths) will make interlaboratory comparison very difficult.

In this paper, we wish to present an alternative parameter as a measure of PAH uptake. 1-Hydroxy pyrene, the main metabolite of pyrene, accounts for a large percentage of the total PAH metabolite profile in bile of fish exposed to combustion-related PAHs (Krahn et al., 1987). The same compound (in urine samples) has been introduced as a marker metabolite for occupational exposure to PAHs (Jongeneelen et al., 1988). Here, we will describe how 1-hydroxy pyrene is easily quantitated in fish bile samples by means of synchronous fluorescence spectrometry (SFS). We will present the validation of the method, and its application to a mesocosm experiment in which the uptake of pyrene by flounder (*Platichthys flesus*) from different sediments and via various uptake routes was investigated. The applicability of the technique to field surveys is discussed.

EXPERIMENTAL

Chemicals

1-Hydroxy pyrene (1-OH pyrene, pyrenol-1) was purchased from the NCI chemical carcinogen repositories (MRI, Kansas City). Uridine diphosphate α-D-glucuronic acid (UDP-GA) was obtained from Sigma. β-Glucuronidase (30 U/ml)/arylsulfatase (20 U/ml) EC 3.2.1.31/EC 3.1.6.1 was supplied by Merck. All chemicals were used without further purification. The solvent used for the spectrofluorimetric measurements (ethanol/deionized water 50:50 v/v) was regularly checked for spectral purity. Analytical grade ethanol from some suppliers contained unacceptable levels of fluorescent impurities, often strongly varying between different batches. Best results were obtained with Merck Uvasol or Ferak analytical grade ethanol.

Origin of bile samples; PAH exposure in mesocosms

Flounders (*Platichthys flesus*) captured at the Wadden Sea were kept during four weeks in large flow-through tanks and exposed to three levels of PAH stress, denoted as mesocosm 1, 2,

and 3 (details in: Hofstraat et al., 1993):
1) Moderately polluted harbor sediment from Rotterdam (dredging class II); food and water equilibrated with the same sediment.
2) Indirect exposure to harbor sediment via food and water that had been equilibrated with mesocosm 1; a relatively clean Wadden Sea sand bottom was present in the basin.
3) Wadden Sea sand (control group); food and water equilibrated with the same Wadden Sea sediment.
Four identical mesocosms were prepared for each exposure level (two different diets, duplicates). Fish were fed either shrimp (*Crangon crangon*) or lugworm (*Arenicola marina*) until two days before section. PAH analysis in whole shrimp by means of HPLC with fluorescence detection (after Soxhlet extraction and chromatographic clean-up over silicagel and florisil) revealed very low PAH body burdens. The concentrations of 4- and 5 ring PAHs ranged from 1 ng/g for benzo[a]pyrene to 10 ng/g for pyrene, and were similar in all mesocosms. The PAH contents of the sediments were determined with HPLC/fluorescence after wet sieving over 63 μ nylon mesh (Klamer et al., 1990) and represent the typical range of PAH levels encountered in the Dutch coastal waters (Harbor sediment: pyrene = 800 ng/g; Wadden Sea sand: pyrene = 180 ng/g dry weight of fine fraction). Bile was taken from the gall bladder by means of a 1 ml syringe and stored in vials at -20 °C until further use. Duplicate determinations after 12 months of storage did not reveal any changes in the samples.

Preparation of pyrene-1 glucuronide

The synthesis of conjugated 1-OH pyrene was based on the glucuronidation reaction described for 3-OH BaP (Boiret and Marty, 1986): 0.5 ml of a 5 x 10^{-4} M solution of 1-OH pyrene in dimethyl sulfoxide was diluted to 250 ml with aqueous phosphate buffer (0.01 M; pH = 7.4; also containing 2 mM of $MgSO_4$). 50 mg UDG-GA was added, as well as ca. 40 mg of flounder liver microsomes. The reaction mixture was stirred at room temperature. At regular intervals the process was monitored spectrofluorimetrically; the emission spectrum of 1-OH pyrene (in ethanol/water 50:50) becomes more intense and shifts to shorter wavelengths upon conjugation. After 100 minutes the reaction was stopped; spectrofluorimetric analysis of the total reaction mixture or of hexane extracts revealed no trace of unreacted 1-OH pyrene. The reaction mixture was diluted with an equal amount of ethanol (precipitation of enzymes and buffer salts) and centrifugated for 10 minutes at 10,000 g. The supernatant was stored at -20 °C until further use.

High performance liquid chromatography

PAH metabolite conjugates were hydrolyzed by addition of 10 μl of β-glucuronidase/ arylsulfatase solution to 25 μl of bile in 1 ml of water (2 hours incubation at 37 °C). The resulting free, less polar metabolites were quantitatively extracted with 4 times 1 ml of hexane; the combined organic fractions were evaporated to dryness and redissolved in 2 ml of ethanol. In the remaining water phase, no significant fluorescence at 1-OH pyrene wavelengths was

detected. In an alternative, time-saving approach the extraction step was omitted: after incubation, 2 ml of ethanol was added to precipitate the enzymes; after centrifugation, the supernatant could be injected directly into the HPLC system. When the second method was used, the sample still contained high levels of polar, fast eluting compounds, but these did not interfere with the quantitation of 1-OH pyrene.

The HPLC system consisted of a Gilson 302 pump, a 20 cm analytical column (3.1 mm i.d.; stationary phase Rosil C18 HL) and a Shimadzu RF 530 fluorescence detector. Excitation/emission wavelengths were 345/ 395 nm (spectral band pass 20 nm). A Schott UG 11 low-pass filter and a Schott WG 375 cut-off filter were fitted to reduce stray-light. The mobile phase was acetonitrile/water (70:30 v/v); the flow rate was 0.8 ml/min.

For quantitation, 13 standard solutions of 1-OH pyrene in ethanol were prepared. Two linear calibration graphs were constructed: from 1 x 10^{-9} M to 1 x 10^{-7} M and from 1 x 10^{-7} M to 1 x 10^{-5} M. Correlation coefficients were 1.000 for both curves. The detection limit (S/N = 3) was 5 x 10^{-10} M, which corresponds to 10 ppb (ng/ml) in the original bile sample. As detector noise was the limiting factor, volume reduction of the final analytical sample would improve sensitivity, but that was not needed for this study. The repeatability of the method was 4 % (5 independent determinations).

Synchronous fluorescence spectrometry

Bile samples were thawed in an ultrasonic bath and diluted with ethanol/water (50:50 v/v), in order to obtain a sufficiently transparent sample (1:500; in some cases 1:2000) After dilution, matrix transmission was > 97 % for all samples. The water/ethanol solvent mixture possesses the appropriate polarity to solubilize all bile constituents and obtain a clear sample. Solutions were not deoxygenated prior to analysis. The fluorescence lifetime of conjugated 1-OH pyrene, unlike that of the parent compound pyrene, is rather short (see below) and the fluorescence intensity does not depend very critically on the presence of oxygen. SFS spectra were measured in a 1 cm quartz cuvette using a Spex Fluorolog 2 spectrofluorimeter, scanning both monochromators simultaneously with a constant wavelength difference ($\Delta\lambda$ = 37 nm), and with excitation/emission band widths of 5 nm. For quantitation, the net peak area from 335-356 nm (excitation wavelengths) was measured. The complete analysis (dilution, measurement, and data processing) took 2-3 minutes per sample. Calibration standards of 1-OH pyrene in ethanol/water were stored in the dark at 4 °C in glass vials with air-tight screw caps. Before use, the solutions were sonicated for 3 minutes.

Fluorescence lifetime measurements

Lifetime measurements were carried out employing a Lambda Physik EMG 101 XeCl excimer laser (308 nm; pulse width 6 ns) as excitation source. Fluorescence from the sample solution was collected at right angle and focussed on the entrance slit of a Zeiss M4QIII monochromator and detected with a fast responding (2 ns) photomultiplier tube (type RCA 1P28;

5 dynodes; terminated to 50 Ω at the oscilloscope; operating voltage, 720 V). The PMT signal was led to a Tektronix 11302 oscilloscope and digitized with a Tektronix DCS01 digitizing video camera. Lifetimes were calculated with the help of laboratory-written deconvolution software.

RESULTS

Predominance of 1-OH pyrene

Analyzing the PAH metabolite profile in bile of English sole (*Parophrys vetulus*) from polluted sites in Puget Sound, Krahn and coworkers observed that 1-OH pyrene is the major metabolite derived from PAHs of four or more rings (Krahn et al., 1987). Although the detectability of 1-OH pyrene is far from optimal at 380/430 nm, the compound still accounts for the largest peak in the HPLC-fluorescence chromatogram. A similar predominance in hydrolyzed bile from flounder (mesocosm 1) is illustrated in Fig. 1: A considerable portion of the total fluorescence intensity of the chromatogram can be attributed to 1-OH pyrene. The compound was unambiguously identified on the basis of its high-resolution fluorescence spectrum in an n-octane Shpol'skii matrix at 28 K (Ariese et al., 1993b). Apparently, the uptake rate of pyrene by flatfish is much higher than that of other 4- or 5-ring PAHs (which were also present in the sediment at comparable levels); the special physico-chemical properties of pyrene result in a high bioavailability to aquatic organisms (Landrum, 1989).

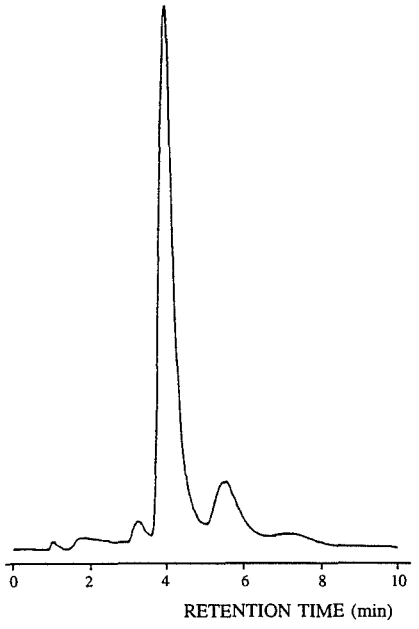

Fig. 1 HPLC separation of flounder bile sample from mesocosm 1, after hydrolysis and extraction with n-hexane. 1-OH pyrene elutes at t = 4 min. Fluorescence detection 345/395 nm.

Synchronous fluorescence spectrometry

The concept of SFS was first introduced by Lloyd (1971), and further developed by Vo-Dinh (1978, 1981). Scanning both the excitation and emission monochromator with a constant wavelength difference (rather than scanning the emission monochromator while keeping the excitation wavelength fixed) results in an important simplification of the fluorescence spectra. In the ideal case, only one single band remains for each compound, which means that spectral interferences are reduced and individual compounds in not overly complex mixtures can be quantitated without chromatographic separation (Vo-Dinh, 1981).

We found that SFS is also well-suited for the determination of 1-OH pyrene in fish bile. Fig. 2a presents a conventional fluorescence emission spectrum of diluted bile of flounder after exposure to Rotterdam harbour sediment, compared to the emission spectrum of a pyrene-1-glucuronide standard solution. The spectral features of conjugated 1-OH pyrene are easily discerned in the total bile spectrum, but correct quantitation is hampered by interferences. Fig. 2b illustrates the advantage of synchronous scanning: the standard spectrum of pyrene-1-glucuronide is almost completely reduced to one single emission band. In the SFS spectrum of total bile, the emission peak attributed to pyrene-1-glucuronide is now more reliably quantitated.

The SFS spectra were recorded with a wavelength interval $\Delta\lambda$ of 37 nm. SFS analysis of parent PAHs in environmental samples is often carried out using a much smaller $\Delta\lambda$ (typically 3-5 nm, corresponding to the compounds Stokes' shift), in order to achieve maximal spectral simplification. The Stokes' shift of conjugated 1-OH pyrene, however, is too small (only 1.5 nm) and the molar extinction coefficient of the 0-0 absorption band is too low for practical application. Using $\Delta\lambda$ = 37 nm, light scattering was strongly reduced and sensitivity was optimal. The repeatability of the method was 9 % (6 independent determinations of a bile sample from the reference mesocosm). The limit of detection (S/N = 3) was 0.1 ppb in the final analytical solution, which corresponds to 50-200 ppb in the original bile sample. The detection limit is directly proportional to the dilution factor, required to obtain a sufficiently transparent matrix. For less deeply colored samples (i.e. in field samples from fish that are not starved prior to section), much lower detection limits can be obtained (10-20 ppb).

Calibration

Obviously, the most straightforward way to perform quantitative fluorimetric measurements would be to use a series of pyrene-1-glucuronide standard solutions for calibration. Unfortunately, this compound is, as far as we know, not commercially available. Another problem is the fact that the conjugate is quite stable at -20 ^{o}C, but at ambient temperatures hydrolysis was observed. For these reasons, we explored the possibility of using free 1-OH pyrene as an alternative standard. From a spectroscopist's point of view, the coupling of glucuronic acid to the phenolic OH-group can be regarded as a minor perturbation of the electronic system of the chromophore. Indeed, the fluorescence excitation- and emission spectra of free- and conjugated 1-OH pyrene are rather similar in shape. Nevertheless, the spectra of the

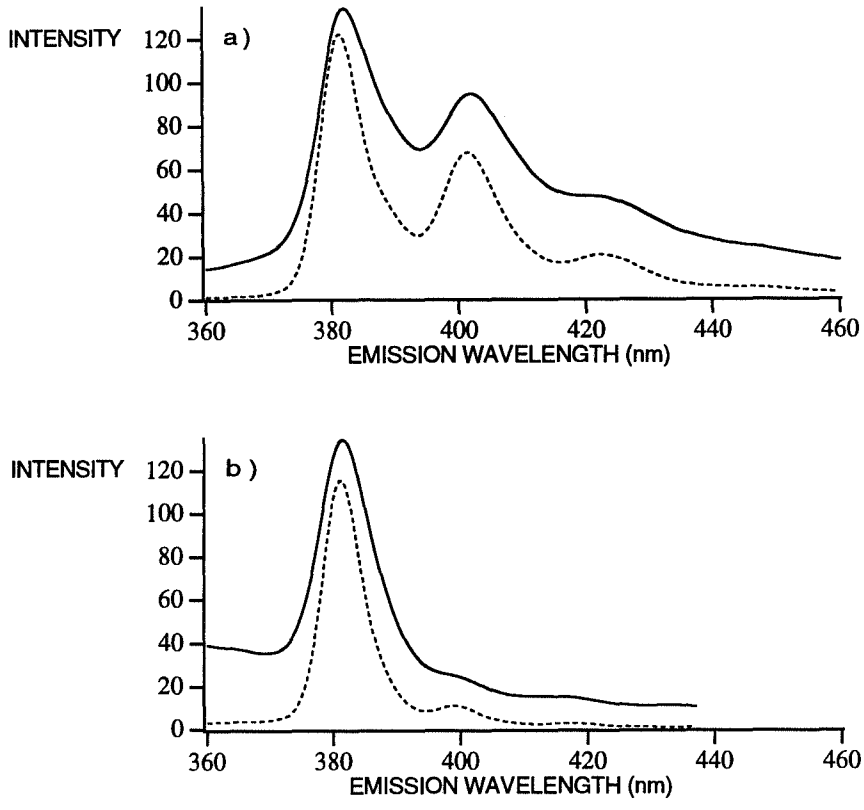

Fig. 2 Conventional (a, λ_{exc} = 345 nm) and synchronous (b, $\Delta\lambda$ = 37 nm) fluorescence spectra in ethanol/water 50:50. Full lines: bile sample from mesocosm 1, diluted 1:2000. Dashed lines: pyrene-1-glucuronide reference standard; 5 x 10^{-8} M.

latter are blue shifted by 5 nm and more intense (Fig. 3). In order to determine the exact difference in fluorescence yield between the two compounds, a number of diluted bile samples and pyrene-1-glucuronide standard solutions (in water) were hydrolyzed with β-glucuronidase/arylsulfatase. Free 1-OH pyrene was quantitatively extracted with 4 times an equal volume of n-hexane. The combined organic fractions were evaporated to dryness in a gentle stream of nitrogen and dissolved in ethanol/water. Compared to the original conjugate solution (1 + 1 diluted with ethanol), the intensity of the main SFS peak had decreased by a factor of 2.2 ± 0.1 (n = 5) (see Fig. 3). Direct spectrofluorimetry of the sample before and after hydrolysis led to similar results, but is less precise because the enzymes added to the solution cause considerable light scatter and thus interfere with the measurement.

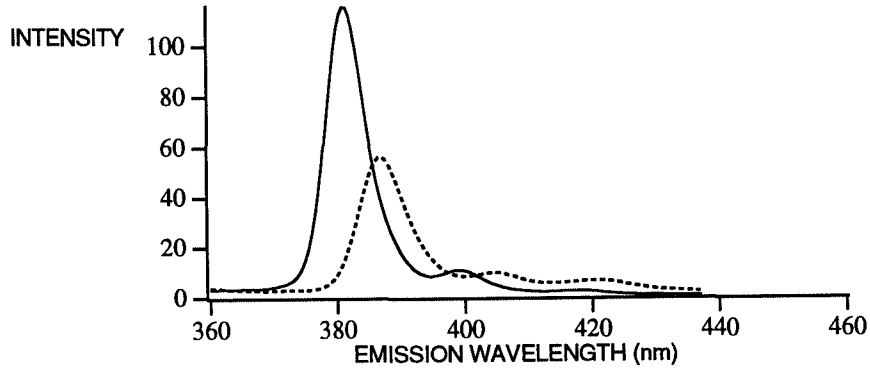

Fig. 3 SFS spectra in ethanol/water 50:50; $\Delta\lambda$ = 37 nm. Full line: pyrene-1-glucuronide conjugate before hydrolysis. Dashed line: 1-hydroxy pyrene (hydrolysis product).

In order to rule out the possibility that some artefact could have caused the decrease in fluorescence intensity, fluorescence lifetime measurements were carried out. According to photochemical theory, the fluorescence quantum yield ϕ of molecules with similar chromophores is directly proportional to the fluorescence lifetime τ (Barltrop and Coyle, 1978). If the observed 2.2-fold decrease in fluorescence yield is correct, the lifetime of free 1-OH pyrene should be shorter than that of the conjugate by the same factor. Using the excimer laser excitation source and the time-resolved detection system described above, the lifetime of free 1-OH pyrene (in ethanol/water, not deoxygenated) was found to be 15 ± 2 ns, and that of the conjugate 29 ± 2 ns, which agrees very well with the relative fluorescence yields determined above. After deoxygenation, the lifetimes were 15 ± 2 ns and 31 ± 2 ns, respectively, which is another indication that fluorescence quenching by dissolved molecular oxygen is not a critical factor.

Validation of the method

1-OH pyrene was determined in a number of flounder bile samples using two independent methods: SFS and HPLC-fluorescence. The concentrations ranged from 280 ng/ml (lowest value from mesocosm 3) to 27.300 ng/ml (highest value from mesocosm 1) Quantitation of the SFS intensities was carried out using standard solutions of free 1-OH pyrene (net peak area from 340-361 nm) and a correction factor of 2.2. Calibration solutions in 500-fold diluted bile from a reference site yielded equal intensities as calibration solutions in clean ethanol/water, indicating that matrix absorption and other possible quenching effects had been sufficiently reduced by dilution. Chromatographic and spectroscopic techniques yielded comparable results; the

concentrations determined with HPLC were on the average 93 ± 17 % (n = 14) of the values determined with SFS. We concluded that the determination of 1-hydroxy pyrene with the rapid SFS technique can be carried out with sufficient accuracy. There seems to be no systematic error (bias), and the imprecision of the method is small compared to the biological variability encountered in this type of samples (see Table I). Accuracy of the SFS method around the detection limit could not be assessed in this study, owing to the relatively high levels in bile samples from the cleanest mesocosm. Recently, a similar intercomparison exercise was carried out with bile samples from a remote North Sea location (55°,00' N; 1°,00' E). 1-OH pyrene concentrations ranged from 230 ng/ml after 2 days of starvation on board to 20 ng/ml in freshly caught fish. The latter value is very close to the detection limit of both techniques, but nevertheless, the discrepancy between SFS and HPLC data never exceeded a factor of two.

Mesocosm study, biomonitoring of pyrene uptake

Bile samples were taken from all individual fish from all mesocosms and were analyzed by means of SFS. The results are presented in Table I. The lowest 1-OH pyrene levels were encountered in bile samples from mesocosm 3 (Wadden Sea sand). 3-4 Times higher concentrations were found in fish indirectly exposed to harbor sediment through water phase and diet (mesocosm 2). Further experiments are needed in order to determine the relative contribution of waterborne PAHs and dietary PAHs to the total uptake. Much higher 1-OH pyrene levels (ca. 20-fold increase compared to mesocosm 3) were observed in fish from mesocosm 1 after direct contact with the harbor sediment.

Table I 1-Hydroxy pyrene and 3-OH Benzo[a]pyrene concentrations in flounder bile as a function of sediment type and diet.

Mesocosm #	1-OH pyrene (shrimp fed)	1-OH pyrene (lugworm fed)	3-OH BaP [a]
1) Harbor sediment direct	13600 ± 5000 (12)	18200 ± 7700 (11)	50 ±36 (9)
2) Harbor sediment indirect	2300 ± 1500 (3)	2700 ± 1600 (13)	7.7 ± 2.4 (4)
3) Wadden Sea sand	820 ± 540 (13)	770 ± 450 (13)	1.2 ± 0.1 (3)

Concentrations in ng/ml bile, expressed as arithmetic mean ± standard deviation (number of samples).

[a] Determined by means of laser excited Shpol'skii spectrometry; 3-OH BaP levels are not classified according to diet; but are combined averages of shrimp fed and worm fed animals (Ariese et al., 1993a).

Direct uptake of sediment-associated PAHs could take place either upon ingestion of particles or via absorption through the skin and gills. It will not be easy to design an experimental setup that could distinguish between these pathways. However, a rough calculation shows that the amount of 1-OH pyrene excreted in the bile (16,000 ppb in ca. 0.25 ml = ca. 4 μg) would correspond to at least 5 grams of ingested harbor sediment. Such quantities were never encountered in the gastro-intestinal tract during dissection. This suggests that, although ingestion of particles cannot be excluded, absorption through skin and gills is probably the most significant uptake route.

No significant differences were found between fish fed shrimp (low fat/low PAH content) and fish fed lugworm (higher levels of accumulated PAHs), which is an extra indication that in the mesocosm system studied the uptake does not take place primarily through the food chain. The amount of 1-OH pyrene excreted in shrimp-fed fish in mesocosm 1 (ca 4 μg) would correspond to an intake of 400 gram shrimp if dietary uptake were the only route of exposure.

DISCUSSION

PAH metabolite analysis in bile is a straightforward method to assess the exposure of fish to PAHs. With respect to sample treatment, fish bile is a more convenient matrix for trace analysis than sediment. Furthermore, bile analysis provides insight into the actual absorption, integrated over all uptake routes, and taking into account bioavailability. As PAHs are rapidly metabolized and cleared from the body (Jimenez, 1987), the biliary metabolite levels reflect the exposure during the last 2-3 days before section. This implies for field monitoring applications that fish migration will usually not be a serious confounding factor, unless the pollution is highly localized. Contrary to other, more general biomonitoring approaches, like the measurement of hepatic enzyme activities (Goksøyr et al., 1991), the PAH-metabolite determination is very specific: exposure to other xenobiotics, like PCB's or dioxins, will not interfere with the measurement. The fact that the method shows an excellent dose-response relationship (Collier and Varanasi, 1991) and that no metabolites will be detected at zero exposure, are important advantages. The wavelengths and offset used for the determination of 1-hydroxy pyrene are very specific for compounds with a substituted pyrene moiety as chromophore (but not pyrene itself). Compounds that could possibly interfere with the analysis are: other conjugates of the same metabolite, like sulfates; conjugates of 4-hydroxy pyrene; conjugated metabolites of alkylated pyrenes; partly saturated metabolites of larger PAHs in which a pyrene-type of chromophore remains, like 7,8,9,10-tetrahydroxy tetrahydro BaP (Vahakangas et al., 1985). Interference from the latter is not likely to be significant, owing to the very low bioavailability of BaP compared to pyrene (see below). Interference from metabolites of alkylated pyrenes could be envisaged in case of petrochemical pollution. Other biliary constituents may display broad-banded fluorescence in the same wavelength area, but are not likely to yield a sharp SFS peak, and will only contribute to the background noise of the measurement.

Recent field studies (section 6.2; Van der Oost et al., 1993; Ariese et al., manuscript in preparation) indicate that the sensitivity of the SFS method will usually be sufficient for field monitoring close to industrialized or urbanized areas. 1-OH pyrene was also easily quantitated in bile of flounder captured along the Dutch coast. Only at more remote North Sea locations, where the pyrene content of the sediment was typically 5 ng/g or lower (Klungsøyr and Wilhelmsen, manuscript in preparation), we found 1-OH pyrene levels close to the detection limit that could not be properly quantitated.

In this experiment, fish were starved for two days prior to section to stimulate the accumulation of metabolites in the gall bladder. During starvation, the metabolite concentrations (as well as the levels of other bile constituents, like biliary pigments) steadily increase, while water is reabsorbed from the gall bladder. In a recent study, in which plaice (*Pleuronectes platessa*), captured at the North Sea, were kept without food for two days in live-tanks on board, 1-OH pyrene levels increased 4-5 fold compared to freshly caught animals (section 6.2; Ariese et al., manuscript in preparation). Similar results were obtained by Collier and Varanasi (1991). These findings indicate that biliary metabolite levels strongly depend on feeding status, which has to be kept in mind when metabolite concentrations, determined after a period of starvation, are to be compared with bile data obtained from freshly captured fish in the field.

In flounder bile the profile of metabolites derived from PAHs containing 4 or more rings is dominated by 1-OH pyrene. A similar predominance was found by Krahn and coworkers in bile samples from other piscine species (1987). This compound could be used as a biomarker for the total uptake of combustion-related PAHs at a particular location. Of course, the use of a single parameter is justified only when the PAH profile of the sediments and the metabolite profile in the bile are more or less constant. Krahn and coworkers (1987) were the first to identify and quantitate a number of specific PAH metabolites in fish bile samples from the field. Recently, we developed a new method (laser excited Shpol'skii spectrometry) for the sensitive determination of 3-hydroxy BaP, the major metabolite of BaP, in bile samples (chapter 5; Ariese et al., 1993a). For comparison, the 3-OH BaP concentrations found in flounder bile samples from the same mesocosms are listed in Table I. The data correlate rather well with the 1-OH pyrene levels determined in this study, but the 3-OH BaP concentrations are lower by a factor of 300-600. This ratio between 3-hydroxy BaP and 1-OH pyrene concentrations is similar to the ratio found in bile of English sole from polluted sites in Puget Sound (Krahn et al., 1987). These findings are a first indication that the PAH metabolite profile could indeed be roughly constant at different locations, and that 1-OH pyrene, which is easily quantitated with SFS, could be used as a relative measure for the total uptake of pyrolytic PAHs. Sometimes there will be reasons to suspect a gross deviation from the usual metabolite profile: for instance strong emission in the SFS spectrum at shorter wavelengths from hydroxy phenanthrenes would be a first indication of significant exposure to creosote or petroleum. In that case more specific techniques like HPLC-fluorescence, GC-MS, or laser-excited Shpol'skii spectrometry could be used to determine the relative contribution of a range of PAH metabolites in more detail.

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6.2

SYNCHRONOUS FLUORESCENCE SPECTROMETRY OF FISH BILE: FIELD STUDIES AT THE SOUTHERN NORTH SEA AND IN DUTCH COASTAL AND INSHORE WATERS

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ABSTRACT

Environmental exposure of fish to polycyclic aromatic hydrocarbons (PAHs) was investigated by measuring a marker metabolite, (conjugated) 1-hydroxy pyrene, in fish bile samples, using synchronous fluorescence spectrometry (SFS). The sensitivity of this rapid technique (limit of detection 10 ng/ml) proved amply sufficient for most locations, except for some remote North Sea sites. Absolute metabolite concentrations depended on feeding status. During several field surveys, some 900 bile samples from 37 locations were collected and analyzed. Bile was collected from flounder (*Platichthys flesus*), plaice (*Pleuronectes platessa*), dab (*Limanda limanda*), whiting (*Merlangius merlangus*), and eel (*Anguilla anguilla*). Different species sampled at the same locality usually showed no important differences in 1-hydroxy pyrene levels. Results indicate that PAH uptake along the Dutch coast is an order of magnitude higher than at the open North Sea. The exposure levels determined for flounder in estuaries and inshore waters were higher than in coastal waters and could probably provide a clue to the spatial patterns of liver neoplasm occurrence. Highest 1-hydroxy pyrene concentrations (up to 9400 ng/ml) were encountered in bile of eel from polluted sites in the vicinity of Amsterdam. The present results on PAH stress in Dutch waters are compared with the data reported for English sole (*Parophrys vetulus*) in Puget Sound, Washington, USA.

INTRODUCTION

Environmental pollution with polycyclic aromatic hydrocarbons (PAHs) may have serious adverse effects on aquatic ecosystems (Varanasi, 1989). Several aromatic compounds such as benzo[a]pyrene are proven carcinogens in rodents, and show similar activity when administered to fish. Upon absorption, PAHs are rapidly metabolized by mixed-function oxygenase (MFO) enzyme systems into a number of hydroxylated derivatives, some of which may form stable adducts with DNA (Varanasi et al., 1986). At several marine and freshwater locations in North America, high prevalences of liver neoplasms were observed in various fish species; high concentrations of PAH metabolites in fish bile from those sites suggested a substantial exposure to PAHs (Krahn et al., 1986; Maccubbin et al., 1988; Johnston and Baumann, 1989).

Several types of fish diseases, including hepatic neoplasms, have also been observed in the North Sea and adjacent waters (reviewed by Vethaak and Rheinallt (1992)). At the southern North Sea and along the Dutch coast, large-scale fish disease surveys have been carried out by the Tidal Waters Division of the Dutch Ministry of Transport and Public Works (Vethaak 1992a; 1992b; Vethaak and Van der Meer, 1991). Flounder (*Platichthys flesus*) and dab (*Limanda limanda*) were chosen as target species; both flatfish species live in intimate contact with the sediments and are susceptible to various diseases. Dab is a common species in the entire North Sea (Rijnsdorp et al., 1993), whereas flounder is very common in the Dutch coastal zone, in brackish estuaries, and in some freshwater areas (Rijnsdorp and Vethaak, 1989). Because of its coastal habitat, flounder is exposed to relatively high levels of pollutants, compared to fish at offshore sites. Vethaak (1992a) reported that the prevalence of liver neoplasms in flounder from Dutch waters showed a consistent spatial pattern throughout the years, with highest levels around stations # 10 and 9 (see Fig. 2). For dab, liver tumor prevalences were lower; spatial patterns and associations with local pollution levels were less pronounced (Vethaak and Van der Meer, 1991).

In order to investigate whether PAH stress could be related to these neoplasia patterns, bile samples from various fish species were collected during two cruises in 1991-1992 at the southern North Sea. Additional flounder bile samples were collected in 1992 in Dutch estuarine and inshore waters, since flounders are known to spend a large part of their younger years in brackish or freshwater areas (Rijnsdorp and Vethaak, 1989).

Circumstantial evidence has also linked PAH pollution to fish health problems in freshwater areas. Extremely high prevalences of liver tumors were observed in brown bullheads (*Ictalurus nebulosus*) from the Black River, Ohio, in the vicinity of a coke plant (Baumann et al., 1987). Studies reporting on the effects of environmental PAHs on other freshwater species are rather scarce. An investigation on PAH exposure levels and effects on fish was started at some freshwater sites around the city of Amsterdam; eel (*Anguilla anguilla*) was selected as the target species. The experiments reported here were carried out as part of a larger study, which included the determination of bioaccumulation levels, enzyme activities, biotransformation rates and the formation of PAH-DNA adducts (Van der Oost, 1993).

Exposure to PAHs was determined by quantitation of a single PAH metabolite (1-hydroxy pyrene (1-OH pyrene) in bile samples. This compound was found to be predominant in bile of fish exposed to PAH-polluted sediments (Krahn et al, 1987), and is readily determined by means of synchronous fluorescence spectrometry (SFS) (section 6.1; Ariese et al., 1993a). Results from Krahn et al. (1987) and Ariese et al. (chapter 5; 1993b) are an indication that the metabolite profile of combustion-related PAHs in fish bile is fairly constant, which would justify the use of a single compound as a marker metabolite for the overall PAH exposure.

In this paper, the main objectives of the different field studies and the results obtained will be separately reported and discussed. Finally, the data will be compared to the levels and effects observed in the American studies in Puget Sound, probably the most thoroughly studied area with respect to PAH-related carcinogenesis in feral fish.

METHODS

Sampling

At the North Sea stations, fish were caught by means of a trawl-net (short, 5-minute hauls). Upon capture, fish were put in tanks containing fresh sea water until further examination. Within an hour, the fish were killed by a blow on the head, measured, and weighed. After dissection, bile was removed from the gall-bladder by means of a syringe, first stored on ice in Nalgene vials or Eppendorf cups, and subsequently stored at -20 °C until further analysis in the laboratory. Stomach and intestine contents were documented, the liver and other internal organs were visually examined for gross pathologies, then prepared for biochemical or histological examination. Only bile samples from fish showing no overt external or internal lesions were analyzed.

At the North Sea stations, fishing usually started around daybreak. It was observed that the flatfish species studied (especially dab and plaice) have quite regular feeding habits: early in the morning most fish caught showed an empty stomach, only partly filled intestine, and full gall-bladder. Later in the morning the majority of fish had a full gastro-intestinal tract and significantly less bile fluid. It was observed that the average bile volume that could be obtained from non-feeding animals was roughly proportional to the weight of the fish. For the SFS analysis, a minimum of 10 µl bile is needed, although larger volumes are more convenient. Sufficiently large volumes (typically 100-400 µl) were usually obtained from the larger fish species (flounder, plaice). On the contrary, most of the dab were rather small (15-20 cm), and often did not possess enough bile for analysis.

In the estuarine and inshore waters, flounder was sampled in a similar way, except that fyke nets were used at some locations. Most of these stations were visited in June 1992; with the exception of stations DO(out), DO(in) and NC, which were sampled in early November 1992.

The freshwater sites around Amsterdam were sampled in July 1991 (approximate water temperature 20 °C at all locations), 10 top-layer sediment samples were collected at each station,

and at least 10 eels were caught with fyke nets. The eels were kept without food for one day to stimulate the buildup of bile and to remove some of the bias caused by different feeding habits (see below). The complete gall-bladder was removed and stored at -20 °C. Unfortunately, some gall-bladders were destroyed during dissection, and some samples became contaminated with blood, resulting in only a limited number of data at some locations. In our experience, removing the bile directly with a syringe when the gall-bladder is still attached to the liver was usually more practical.

Synchronous fluorescence spectrometry

A detailed description of the SFS method has been given in section 6.1 (Ariese et al. 1993a). Samples from the first North Sea cruise, flounder bile samples from estuarine sites and eel bile samples were usually diluted 1:500 with ethanol/water 50:50. Samples from the more remote North Sea locations, as well as most samples from the second North Sea cruise, generally contained lower metabolite levels and were diluted 1:200. Exceptionally dark samples were diluted 1:2000 in order to reduce matrix absorption. Absorption spectra were recorded for some of the darkest samples (after dilution); the approximate matrix transmission T in a 1 cm fluorescence cuvette (right-angle geometry) was calculated from the absorption $A_{\lambda ex}$, $A_{\lambda em}$ of the matrix at the excitation- and emission maxima of the analyte (0.5 cm path length) according to:

$$T_{total} = 10^{-A345} \cdot 10^{-A382}$$

(adapted from Leese and Wehry, 1978). Matrix absorption was considered sufficiently reduced if $T_{total} > 0.97$.

Fluorescence spectra were recorded in the synchronous scanning mode, using $\Delta\lambda = 37$ nm (excitation and emission band widths 5 nm). The solvent blank (mainly Raman scattering) was subtracted from the sample spectra. Quantitation was carried out using a series of 1-OH pyrene standard solutions in diluted bile from a reference site, correcting for the difference in fluorescence quantum yield between free and conjugated 1-OH pyrene as described in section 6.1 (Ariese et al., 1993a). The SFS spectra of the samples were recorded using either a Spex Fluorolog (1st North Sea cruise and eel samples) or a Perkin Elmer LS 50 spectrofluorimeter (2nd North Sea cruise and flounder bile samples from estuarine sites). The detection limit after 1:200 dilution was 10 ng/ml with both instruments. In a limited number of samples, most often bile from dab, the detection limit was somewhat higher (20 or 30 ng/ml) as the result of fluorescent matrix interferences. To validate the SFS method, a limited number of bile samples from the North Sea was also enzymatically hydrolyzed, extracted and analyzed by means of HPLC/fluorescence (see section 6.1; Ariese et al. (1993a) for experimental details). The HPLC method is more time-consuming, but offers better selectivity and sensitivity. For 1-OH pyrene, both techniques yielded comparable results. Before starting the analyses for the second North Sea cruise, some samples from the first survey, that had been stored for a year at -20 °C, were thawed, diluted, and analyzed for a second time to check for interlaboratory consistency.

Reproducibility was good: although the concentrations measured were rather close to the detection limit, the second set of values corresponded to 101 ± 11 % (n = 9) of the first results. Apparently, the use of different instruments or a different set of calibration dilutions had not led to gross systematic errors.

Fish starvation

At the North Sea locations 6 and 12, a separate experiment was carried out to investigate the accumulation of metabolites in bile. A number of plaice was kept in a flow-through tank on board (without food). Some sediment from the same location was also added to the tank. Fish were sacrificed directly, and after 12, 24 and 48 hours of starvation. Bile was collected and analyzed as described above.

Sediment analysis

PAH determination in total sediment was carried out at the laboratory of the OMEGAM environmental institute of the City of Amsterdam. Samples were dried and Soxhlet extracted with hexane/acetone (1:1), according to Van der Oost et al. (1991). After two column chromatographic clean-up steps over aluminum oxide and silicagel, the sample extracts were analyzed by means of HPLC, using a Supelcosil LC-PAH C18 column, a linear gradient from 55-100 % acetonitrile, and fluorescence detection. Peak identification was confirmed by means of GC-MS (details in Van der Oost et al., 1991).

RESULTS AND DISCUSSION

Influence of feeding status

At two North Sea stations it was investigated how the metabolite levels in bile depend on the feeding status of the fish. This was done in order to be able to compare the metabolite concentrations determined in flounder bile in a mesocosm study after two days of starvation (section 6.1; Ariese et al., 1993a) with the metabolite levels encountered in the field in freshly captured fish. Plaice were kept in a flow-through tank and sampled after given periods of starvation. Figure 1 shows the steady increase in metabolite concentration with time; PAHs are continuously being transformed into hydroxylated metabolites, while the total gall bladder volume remains approximately constant as water is being reabsorbed (Collier and Varanasi, 1991; Grossbard et al., 1987). After two days the 1-OH pyrene levels had increased 5-6 fold compared to the average concentrations found immediately upon capture. A similar effect (6-7 fold increase after two days) was observed in flounder from the VISEX mesocosm facilities at Texel (F. Ariese, unpublished results). Obviously, some interindividual variation in 1-OH pyrene concentrations can be expected if the time passed since the last feeding is not the same for each fish. A short starvation period (two days) on board would remove some of that bias, while at the same time the analyte concentrations would increase. That approach could probably lead to a

lower biological variability and better measurement precision, provided the fish survives the starvation period in good health. Unfortunately, the method cannot be combined with measurements of enzyme activity in the liver of the same fish, as the latter should be performed on freshly caught animals. On the other hand, direct section upon capture has some logistic advantages, but sufficient bile is not always available, and the feeding history can only be roughly estimated on the basis of the stomach/intestine contents.

The data presented in this study are obtained without starvation on board. We attempted to correct for the accumulation effect by ratioing the metabolite concentrations with the concentrations of biliary pigments (bilirubin or biliverdin), as suggested by Collier and Varanasi (1991). However, for the field data reported here, that approach did generally not result in a reduction of the statistical spread. We observed that even in a flounder population under controlled laboratory conditions with regular daily feeding, the biliary pigment concentrations varied considerably between individuals after the same starvation period (Ariese, unpublished results). It was concluded that ratioing may introduce an extra source of variability, and is only useful if the differences in accumulation level are very large.

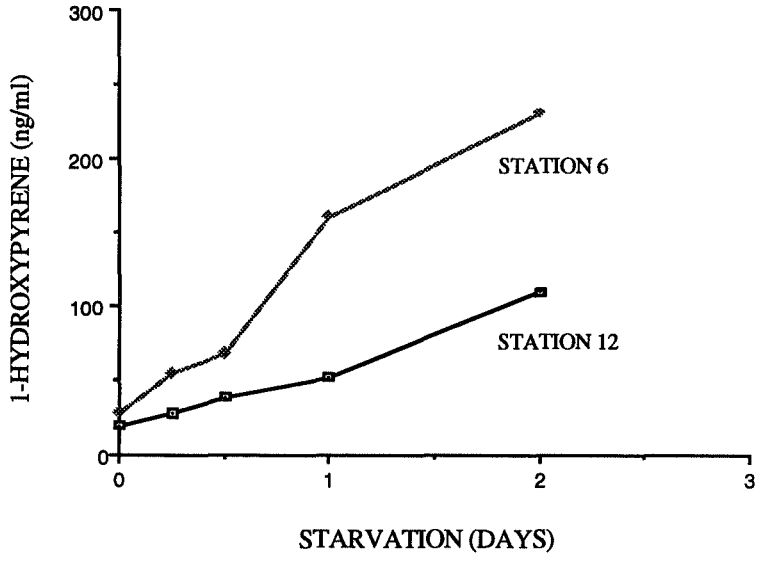

Fig. 1 Accumulation of 1-OH pyrene in bile of plaice during starvation on board; point t = 0 represents fish freshly caught with a full stomach; point t = 0.25 is arbitrarily assigned to animals captured with an empty stomach and partly filled intestinal tract.

North Sea

Introduction Within the framework of the Dutch INP (Integrated North Sea Programme) microcontaminants program, we participated in two marine cruises with the Research Vessel "Pelagia" organized by the Netherlands Institute for Sea Research (NIOZ). The first cruise took part in late summer (Aug 26-Sept 14, 1991), and included 16 stations in the southern North Sea. (Fig. 2) . Most of these stations, as well as some new ones, were sampled during the second cruise in late spring (May 18-June 6, 1992) (see Fig. 3).

Over a dozen of scientists from different research institutes from the Netherlands, Norway and the USA, representing many different disciplines, took part in the program. Various parameters were studied, ranging from contaminant levels (polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dioxins and organotin compounds) in sediments and tissues, through PAH metabolite levels and enzyme activities to pathology. In this section, however, we will confine ourselves to the determination of 1-OH pyrene in fish bile.

Results The 1-hydroxy pyrene content of several hundred bile samples (from all specimens captured at all locations) was determined by means of synchronous fluorescence spectrometry (SFS). The method was found to be very rapid and practical (3 minutes per sample; no sample preparation needed except for a dilution step). At some clean stations at open sea (e.g. # 5, 12) the metabolite concentrations were very close to the detection limit. Quantitation at very low levels was sometimes hampered by spectral overlap with the (low) native fluorescence of other bile constituents (mainly observed for dab bile). At most stations, however, biliary 1-OH pyrene could be accurately measured with the SFS method.

Table I lists the mean concentrations of 1-OH pyrene in bile at the 16 stations visited during the first cruise. At only a few locations (e.g. #4), there appears to be some interspecies difference, but that is not generally observed at other stations. In fact, it rather seems that the daily PAH uptake is more or less the same for all bottom-dwelling species investigated. Therefore, the last column of Table I lists the pyrene metabolite concentration averaged over all fish captured at a given location. These average concentrations are also displayed in Fig. 2.

Table I Concentrations of 1-hydroxy pyrene in fish bile (first cruise).

Loc.	Dab	Whiting	Flounder	Plaice	Combined
2	130 ± 60 (6)		170 ± 70 (11)		160 ± 70 (17)
3	50 ± 30 (7)	40 ± 40 (5)		80 ± 40 (9)	60 ± 40 (21)
4	40 ± 20 (4)	130 ± 60 (5)		90 ± 30 (8)	90 ± 50 (17)
5	~10 (6)			~20 (3)	~10 (9)
6	20 ± 10 (8)	40 (2)		40 ± 20 (14)	30 ± 20 (24)
7	150 ± 60 (3)			120 ± 40 (10)	130 ± 50 (13)
8	60 ± 10 (3)	120 ± 50 (4)		120 ± 40 (5)	110 ± 40 (12)
9	20 ± 10 (7)		20 ± 10 (4)	60 ± 50 (15)	40 ± 40 (26)
10			240 (1)	120 ±130 (6)	140 ±130 (7)
11	170 ± 70 (6)		260 ±100 (9)	260 ±140 (6)	240 ±110 (21)
12	20 ± 20 (3)			20 ± 10 (16)	20 ± 10 (19)
13	~10 (6)	20 (2)		60 ± 40 (18)	50 ± 40 (26)
14	70 ± 60 (3)	20 (1)	120 ± 10 (3)	120 ± 70 (3)	90 ± 50 (10)
15	~10 (2)			30 ± 10 (10)	30 ± 10 (12)
16		20 (2)		30 ± 20 (3)	30 ± 20 (5)
17	50 ± 10 (3)	40 (1)	30 (2)	80 ± 40 (11)	70 ± 40 (17)

Concentrations in ppb (nanogram /ml bile).
Data are expressed as the arithmetic mean ± standard deviation (number of samples).

Some trends can be discerned in Fig. 2: highest PAH exposure levels are found in the coastal area, especially around the harbors of Rotterdam (#11) and IJmuiden (#10), and near the Western Scheldt (#17, 14); elevated pyrene uptake is also observed north of the Wadden Sea and at the Frisian Front (#8, a deposition area of Rhine sediment). Much lower PAH metabolite levels are found around the Dogger Bank (#5 and 6) and also the area close to the English Channel seems relatively unpolluted, as it is constantly flushed with relatively clean water from the Atlantic Ocean. These spatial patterns indicate that at the North Sea the distribution of pyrene and related PAHs of pyrogenic origin, (but probably not petroleum PAHs) is mainly governed by river output of polluted sediment. The plume of the river Rhine does not stretch out far into the open sea; the sediment-associated PAHs are transported to the North along the coast by the prevailing currents (Eisma and Irion, 1988). Atmospheric deposition or shipping-related pollution would result in a more diffuse pattern.

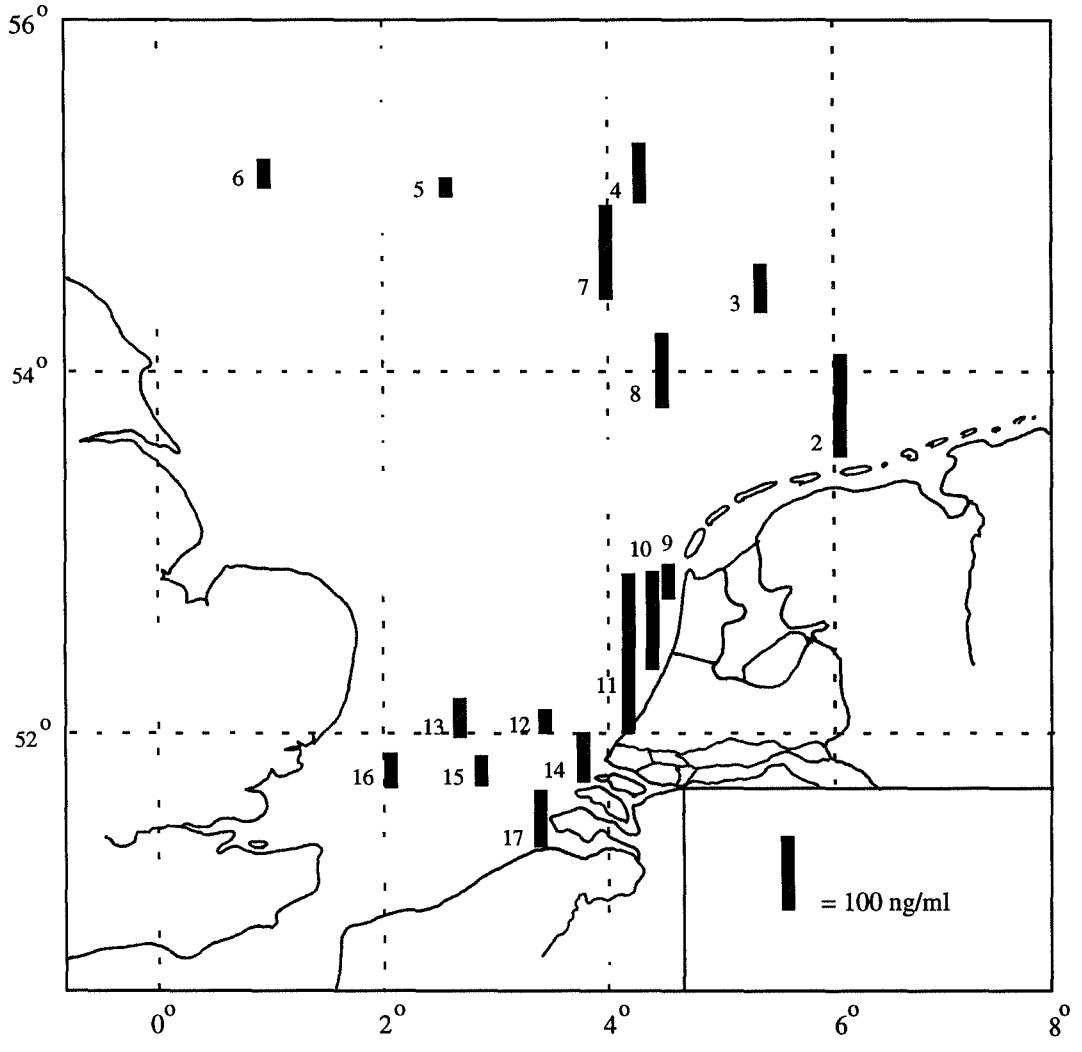

Fig. 2 Sampling stations at the North Sea and average 1-OH pyrene concentrations in bile of bottom-dwelling fish; first cruise , Aug-Sept 1991.

Table II Concentrations of 1-hydroxy pyrene in fish bile (second cruise).

Loc.	Dab	Flounder	Plaice	Combined
2	~30 (n = 26)	70 ± 40 (n = 20)	40 ± 10 (n = 14)	50 ± 30 (n = 60)
4	20 ± 10 (n = 15)		30 ± 10 (n = 12)	30 ± 10 (n = 27)
5	~10 (n = 26)		~20 (n = 9)	~10 (n = 35)
6	~20 (n = 8)		20 ± 10 (n = 11)	20 ± 10 (n = 19)
7	20 ± 20 (n = 15)		30 ± 10 (n = 12)	30 ± 20 (n = 27)
8	30 ± 10 (n = 12)		40 ± 10 (n = 6)	30 ± 10 (n = 18)
9	40 ± 10 (n = 9)	90 ± 40 (n = 12)	30 ± 10 (n = 9)	60 ± 40 (n = 30)
10	70 ± 20 (n = 21)	140 ± 50 (n = 11)	70 ± 20 (n = 12)	90 ± 40 (n = 44)
11	100 ± 30 (n = 15)	160 ± 40 (n = 12)	140 ± 40 (n = 14)	130 ± 40 (n = 41)
19	~10 (n = 4)		~20 (n = 9)	~20 (n = 13)
20	~10 (n = 6)		~10 (n = 12)	~10 (n = 18)
21	~10 (n = 11)		30 ± 10 (n = 15)	20 ± 10 (n = 26)
22	~10 (n = 9)		~10 (n = 12)	~10 (n = 21)
23	~20 (n = 13)	110 ± 40 (n = 20)	20 ± 10 (n = 6)	70 ± 50 (n = 39)
24	30 ± 10 (n = 11)	70 ± 20 (n = 12)	80 ± 20 (n = 12)	60 ± 30 (n = 35)

Concentrations in ppb (nanogram /ml bile).
Data are expressed as the arithmetic mean ± standard deviation (number of samples).

The average 1-OH pyrene concentrations determined in bile from the second North Sea cruise are presented in Table II and Fig. 3. Again, no clear differences were observed between dab and plaice, but 1-OH pyrene concentrations in flounder bile were higher at some locations (# 9, 10, 23). This difference was probably caused by different feeding habits: Most dab and plaice were found with some food rests in stomach or gut, while most flounders captured at these locations were found with an empty stomach.

Interestingly, although figures 2 and 3 show very similar spatial trends, the absolute values determined in the samples from cruise 2 are lower by a factor of 2-4. The difference could probably be due to a seasonal effect: during cruise 2 in late spring, the average water temperature at the sea bottom was 5-6 degrees lower than during cruise 1. Jimenez et al (1987) reported that the uptake of benzo(a)pyrene by the bluegill sunfish (*Lepomis macrochirus*) is almost 6-fold slower when the fish are kept at 13 °C instead of 23 °C. Of course, the metabolite content in fish bile should be regularly monitored in different seasons during several years to investigate whether the PAH uptake rate is indeed a reproducible function of the water temperature.

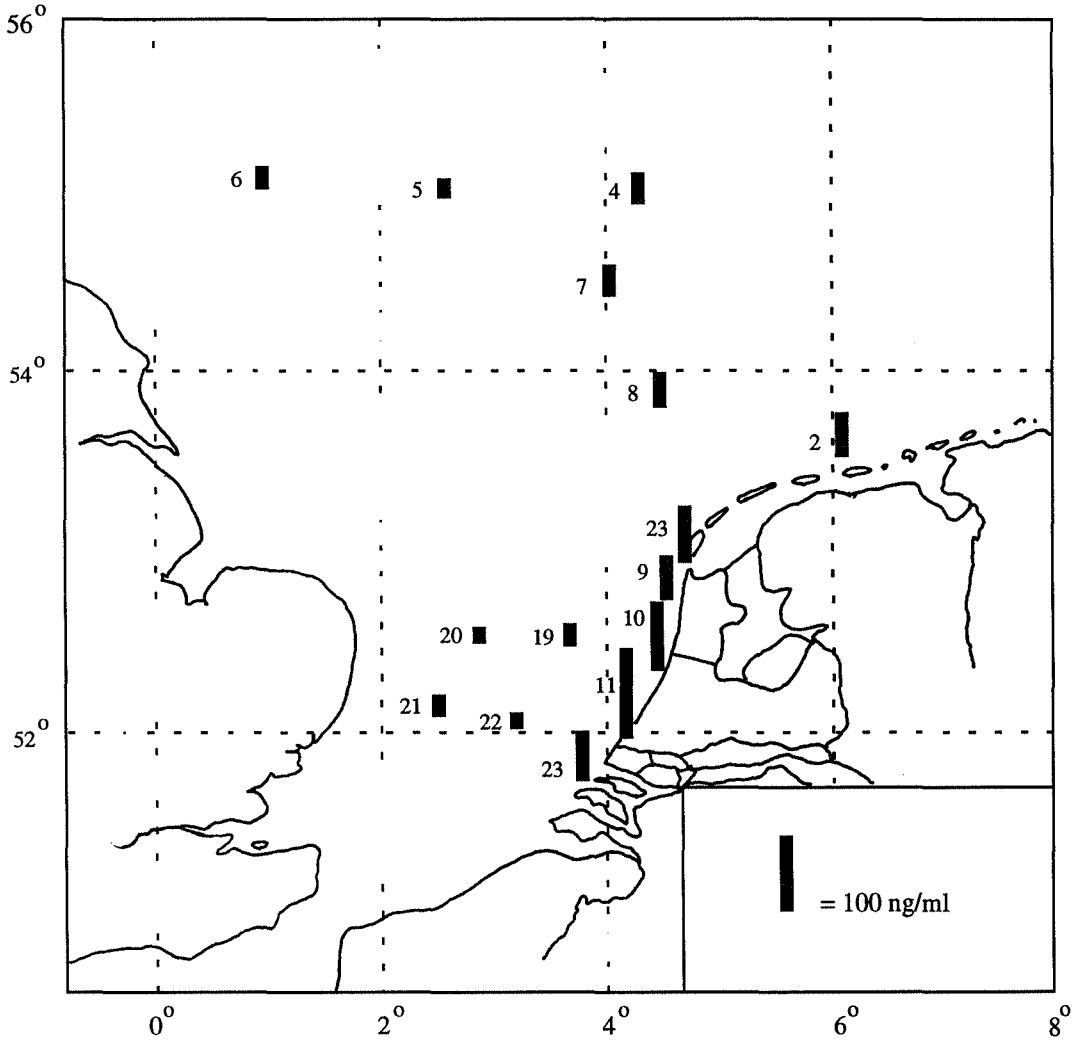

Fig. 3 Sampling stations at the North Sea and average 1-OH pyrene concentrations in bile of bottom-dwelling flatfish; second cruise , May-June 1992.

Dutch estuaries

Introduction As mentioned above, monitoring studies at the North Sea (Vethaak 1992a) revealed high prevalences of liver neoplasms in flounder in coastal areas, especially around stations # 9, 10, and 11 (Fig. 2). The risk of developing liver neoplasms was also found to be a steep function of age, with locally prevalences up to 30 % for the 6+ age group (a similar age effect was noted by Baumann et al. (1987)). Interestingly, signs of liver neoplasia were only very rarely observed in fish captured in the Western Scheldt and Ems-Dollard estuaries (Vethaak, 1992a); sites that are known to be more heavily polluted than the Dutch North Sea coast, but where predominantly younger fish occurs. Interpretation of these epidemiological findings is difficult, especially because the effects (liver neoplasms) develop very slowly (Vethaak and Rheinallt, 1992). The migratory behavior of the particular fish species should be thoroughly studied. Dutch tagging studies (Rijnsdorp and Vethaak, 1989) have revealed that most flounders grow up in inshore waters. In the spawning season (December-April), mature flounder swim out to the open sea, but usually come back to the same estuary in early spring and migrate very little during the summer. This behavior is important for the understanding of the spatial and seasonal patterns observed during fish disease surveys: flounder sampled outside the spawning season (preferably late summer) can be considered representative for the area of capture. Older fish, however, do usually not enter the freshwater area, but remain in the coastal zone, close to the mouth of the estuary. Vethaak (1992a) hypothesized that the high prevalence of liver neoplasms, observed in older fish captured outside an estuary, could be related to exposure to high levels of pollutants within the freshwater area during earlier years. Furthermore, high prevalences of liver neoplasia were recently observed in young flounder captured in the North Sea Canal (Vethaak, 1991), an intensive shipping route boarded by several industries in the Amsterdam harbor area and a large steel production plant at the North Sea mouth.

For these reasons, it was decided that next to the PAH-exposure data from the North Sea, 1-OH pyrene levels should also be determined in bile samples from those estuarine and freshwater areas that are known nursery areas of flounder.

Results Names and characteristics of the sampling sites, as well as the results of the SFS measurements are compiled in Table III and indicated in the map of Fig. 4. Combining these data with the results obtained at the North Sea (see previous section), a pollution gradient is observed along the Western Scheldt (stations SA, VL and 17) and along the Ems-Dollard estuary (stations DL, BW and 2).

Table III Coastal and freshwater sampling sites, and 1-OH concentrations in flounder bile.

Code	Sampling site	1-OH pyrene in bile	
DL	Dollard estuary	290 ± 150	n = 7
BW	Bocht van Watum, mouth of Ems-Dollard estuary	260 ± 100	n = 4
WS	Wadden Sea (Blauwe Slenk)	130 ± 80	n = 21
HA	Wadden Sea, north of Harlingen	120 ± 70	n = 12
DO(out)	Den Oever sluices Wadden Sea side	100 ± 70	n = 17
DO(in)	Den Oever sluices freshwater side (Lake IJssel)	90 ± 40	n = 21
NC	North Sea Canal, 5 km inland	1900 ± 900	n = 20
VL	Mouth of Western Scheldt estuary near Vlissingen	150 ± 100	n = 22
SA	Saaftinge, mouth of Scheldt River	580 ± 300	n = 21

Concentrations in ppb (ng/ml bile).
Data are expressed as the arithmetic mean ± standard deviation (n = number of samples).

No important differences in PAH stress are observed within the Wadden Sea area. Prevalences of liver neoplasia are rather low at the Wadden Sea. Frequent reports of various external diseases in flounder at the seaside of the freshwater drainage sluices at Den Oever (station DO(out)) can be related to other environmental stress factors (Vethaak 1992b), but do not seem to be linked to PAH pollution. Very high 1-OH pyrene concentrations were observed in bile of fish from the North Sea Canal. Extreme PAH exposure could probably explain the (unusual) prevalence of liver neoplasms in 2-and 3-year old flounder in that area. Liver neoplasms found in older flounder along the North Sea coast, especially around stations 10 and 9, may be related to recent exposure in the coastal zone, but given the high 1-OH pyrene levels determined in flounder from the North Sea canal (station NC), one could argue that PAH uptake in inshore waters at an earlier age might also be an important factor.

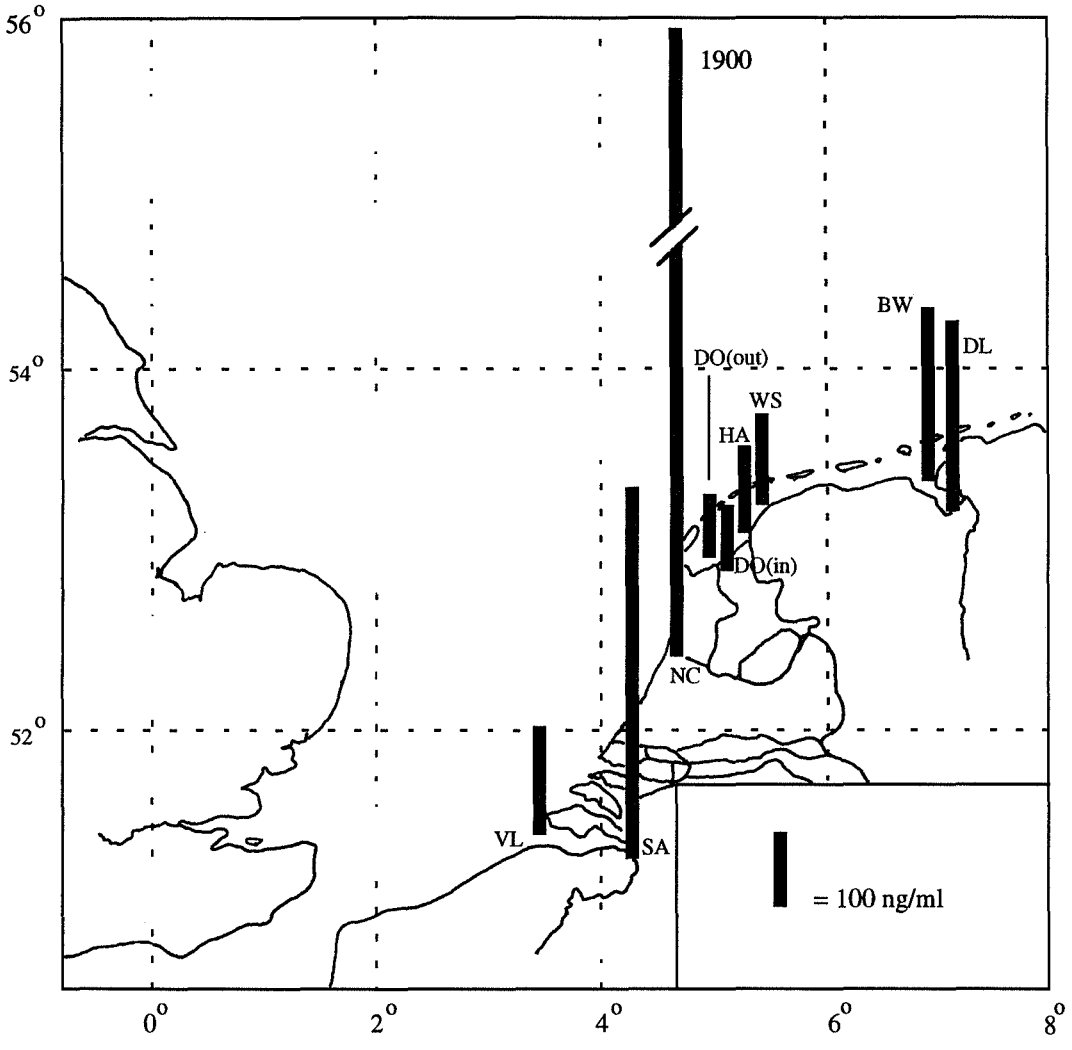

Fig. 4 Coastal and inshore sampling sites, and 1-OH pyrene concentrations in flounder bile.

Freshwater sites around Amsterdam

Introduction In this section the determination of 1-hydroxy pyrene in bile of eel from a number of polluted sites in and around the city of Amsterdam is reported. The experiments were carried out as part of a multidisciplinary study on the effects (bioaccumulation, enzyme induction, formation of DNA adducts) of PAHs in the freshwater environment; the target fish chosen was eel. Eel is common in most Dutch freshwater areas, also at more polluted sites. Because of its commercial value, data on local occurrence, migratory patterns, and optimal fishing methods are readily available. Furthermore, as is the case for the flatfish species studied, eel is a benthic feeder, and is therefore expected to be maximally exposed to sediment-associated pollutants. The program was coordinated by OMEGAM; the overall results are reported in a separate paper (Van der Oost et al., 1993). In this section we confine ourselves to the analysis of 1-hydroxy pyrene in fish bile; correlations with pyrene levels in the sediments are discussed.

Six freshwater sites around the city of Amsterdam were selected (see Fig. 5):

GP Gaasperplas; a relatively unpolluted recreational lake (no motorboats allowed); this lake will be used as a reference site.

DZ Diemerzeedijk; located near a hazardous waste landfill; recently, the heavily polluted sediments were covered with a 20 cm layer of clean sand.

NM Nieuwe Meer; a moderately polluted lake, former site for the discharge of dredging material from the city canals of Amsterdam.

AH Amerika Haven; a moderately polluted harbor, present site for the discharge of dredging material from the city canals of Amsterdam and of industrial fly ash.

IJ Afgesloten IJ; enclosed part of the former IJ-harbor, polluted with industrial waste.

VM Volgermeerpolder; ditches surrounding a former landfill North of Amsterdam, locally heavily polluted with hazardous chemical waste.

Results The pyrene content of the sediments and the results of the SFS measurements in eel bile are summarized in table IV. The data indicate that the use of Lake Gaasperplas (GP) as a relatively clean reference site seems justified. PAH exposure is even lower at station DZ; the Diemerzeedijk used to be one of the most heavily contaminated sites in Amsterdam, but as a provisional measure prior to a more permanent solution, the polluted sediment had recently been covered by a layer of clean sand. The pyrene content in the coarse-grained sediment samples from DZ was close to or below the detection limit of 0.1 mg/kg. The low 1-OH pyrene concentrations in eel bile from DZ indicate that (at least temporarily) the measures taken at the Diemerzeedijk significantly reduce PAH stress.

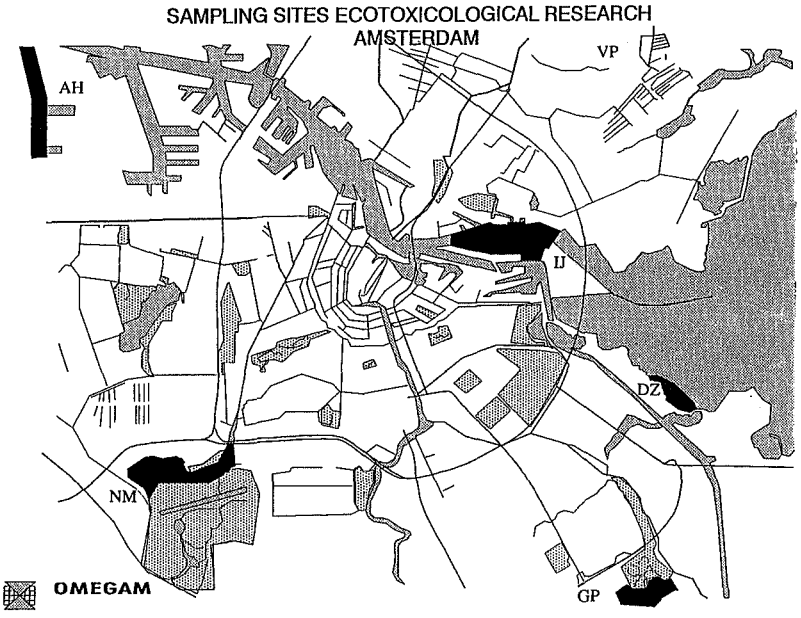

Fig. 5 Map of greater Amsterdam, indicating sampling sites.

Table IV Pyrene contents in sediments and 1-OH pyrene concentrations in bile of eel.

Site		pyrene in sediment mg/kg dry wt	1-OH pyrene in bile µg/ml
GP	Gaasperplas	0.3 ± 0.3 (n = 10)	1.3 ± 1.3 (n = 8)
DZ	Diemerzeedijk	ca. 0.1 (n = 10)	0.9 ± 0.2 (n = 5)
NM	Nieuwe Meer	3.0 ± 2.0 (n = 10)	9.4 ± 7.2 (n = 4)
AH	Amerika Harbor	1.3 ± 0.7 (n = 10)	4.8 ± 1.2 (n = 8)
IJ	Afgesloten IJ	1.4 ± 0.8 (n = 10)	4.5 ± 3.5 (n = 3)
VM	Volgermeerpolder	5.5 ± 2.5 (n = 10)	2.0 ± 3.0 (n = 6)

Data are expressed as the arithmetic mean ± standard deviation (n = number of samples).

At stations AH, IJ and NM, the 1-OH pyrene levels showed a proportional increase with the average pyrene content of the sediments (see Fig. 6). At the Volgermeerpolder (VM), however, the average PAH uptake was only slightly enhanced compared to the reference site, not reflecting the high PAH levels in the sediment samples. Furthermore, the statistical spread was unusually high. A possible explanation for that discrepancy could be the fact that the pollution is highly localized around the former landfill; as the result of fish migration, the actual exposure of the fish could have been much lower. The relatively low DNA-adduct levels in eel liver homogenates from the Volgermeerpolder site (Van der Oost et al., 1993) are consistent with that hypothesis.

As a result of the high elimination rates for PAHs in teleost fish (Jimenez et al., 1987), PAH metabolite levels in fish bile only reflect the most recent uptake. Therefore, fish migration will not be a serious confounding factor as long as the pollution is more or less equally distributed over an area of the same order of magnitude as the daily migration distances. If information is needed on PAH exposure levels around a specific point source, migration problems can be avoided by using caged fish (Oikari and Kunnamo-Ojala, 1987) or mussels (Dunn and Stich, 1976; Boom, 1987).

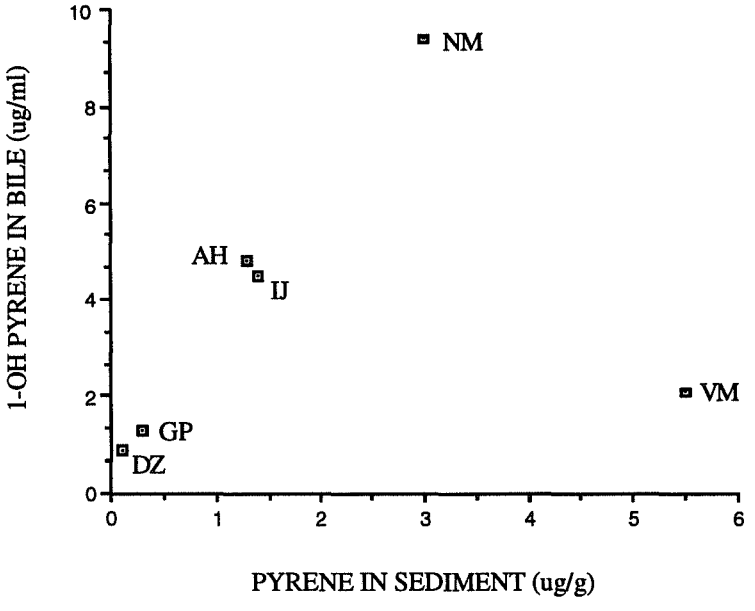

Fig. 6 1-OH pyrene concentrations in eel bile versus pyrene content of sediment.

COMPARISON WITH AMERICAN STUDIES

Most field data on biliary metabolite concentrations are available for English sole (*Parophrys vetulus*) from more or less polluted sites in Puget Sound, Washington (Krahn et al., 1986; 1987). For over a decade, a large number of field and laboratory studies has been carried out in the Puget Sound area to investigate the effects of environmental carcinogens like PAHs, and their relevance to fish health in the field (Myers et al., 1990). Unfortunately, the 1-OH pyrene concentrations summarized above (Tables I to IV) cannot be directly compared with the Puget Sound data, since different quantities are measured. In the American studies the biliary metabolite levels are quantitated by integrating the total signal intensity of an HPLC chromatogram, using fluorescence detection and excitation/emission settings of 380/430 nm. The resulting area, often expressed in "BaP equivalents", is a relative measure for all biliary compounds (mainly PAH metabolites) that show some fluorescence response at that wavelength combination. The quantity should be related to the 1-OH pyrene concentration, assuming a more or less constant metabolite profile (Krahn et al., 1987; Ariese et al., 1993b), and knowing that 1-OH pyrene itself is a major contributor to the total fluorescence. Although a lack of reliable data precludes the determination of a conversion factor with a satisfactory degree of certainty, one can conclude from the paper by Krahn and coworkers (1987) on individual metabolite concentrations in five samples, that the data in "BaP equivalent" units are probably an underestimation of the 1-OH pyrene concentrations. Krahn and coworkers (1986) reported average biliary fluorescence levels between 270 and 2100 ng/ml BaP equivalents for English sole captured at polluted harbors and waterways where enhanced prevalences of various hepatic lesions, including neoplasms, are regularly observed. Metabolite fluorescence levels ranged from 70-100 ng/ml BaP eq. at reference sites, where such lesions were absent or only rarely observed in older fish (Malins et al., 1988). Comparing these figures with the 1-OH pyrene concentrations determined in the Netherlands, we conclude that PAH uptake at the offshore North Sea sites is very low. At the coastal and estuarine stations, the metabolite concentrations are comparable to those found in the reference areas in Puget Sound. The biliary metabolite concentrations determined in the North Sea Canal and at most locations around Amsterdam are of the same order as the levels encountered at the most polluted sites in Puget Sound.

SUMMARY AND CONCLUSIONS

The applicability of biliary 1-hydroxy pyrene as a marker metabolite for PAH stress in the aquatic environment was investigated. Synchronous fluorescence spectrometry proved to be a very rapid and convenient method for the quantitation of this compound in bile from various fish species. The sensitivity of the method was amply sufficient for most field locations, except for some remote stations at open sea. In comparison with HPLC determination, the SFS method provides little information on other metabolites present in the sample, but the analysis time is

much shorter. The sensitivity for 1-hydroxy pyrene is slightly better with HPLC.

Contrary to the more conventional analysis of parent PAHs in sediment, the measurement of biliary metabolites provides direct insight into the amount of PAH that has actually been absorbed by the organism, taking into account all possible uptake routes. This proved particularly important at some North Sea locations: at stations #10 and 11 the PAH levels in the coarse-grained sediments were extremely low (pyrene content ca. 1 ng/g; Klungsøyr and Wilhelmsen, manuscript in preparation), but bile analysis revealed a substantial pyrene uptake, presumably originating from suspended matter.

The North Sea data reported here indicate that pyrene exposure is mainly related to river output of polluted sediment (Rhine, Meuse, Scheldt), which is subsequently transported to the north along the coast (Eisma and Irion, 1988). Similar distribution patterns have been reported for other sediment-associated contaminants, like polychlorinated biphenyls and heavy metals (Kramer et al., 1990). The spatial pattern of PAH metabolite concentrations in bile of flatfish is very similar to that of accumulated PAHs in mussels (Boom, 1987).

Compared to the North Sea, PAH exposure levels were enhanced in the Ems-Dollard and Western Scheldt estuaries; highest levels in flounder were observed inshore in the North Sea Canal. 1-OH pyrene concentrations in bile of eel from freshwater sites around Amsterdam were even 1-2 orders of magnitude higher than those observed in flatfish bile at marine stations. A good correlation was found with the pyrene content of the sediment; only around the Volgermeer landfill, the average metabolite concentration was lower than expected,which could probably be due to migration. PAH metabolism and excretion rates are fairly high in fish (Jimenez et al, 1987), which means that metabolite concentrations will reflect only the most recent exposure, and fish migration is usually not a serious problem. The method should not be expected, however, to yield accurate information on PAH pollution levels around point sources, as was also pointed out by Krahn et al. (1986).

Biliary metabolite levels increased steadily when fish were kept without food; differences in feeding status can be a major confounding factor. It should be investigated whether keeping the fish without food for some days in live tanks on board, or analyzing only fish captured with an empty stomach, would be a practical way to reduce variability. In this study, ratioing with the concentration of biliary pigments generally did not result in a lower biological spread.

At most North Sea stations, there appeared to be no systematic differences in pyrene uptake between the three flatfish species studied; also 1-OH pyrene levels in the bottom-dwelling gadoid species whiting was more or less the same (small number of observations).This illustrates the general applicability of the method. Sufficient bile (> 10 µl) could be collected from most larger fish (heavier than 100 g). Highest yields were obtained at daybreak. Field surveys like these should be carried out outside of the spawning period. Because of an apparent seasonal effect (compare Figs. 2 and 3), stations that are to be compared should preferably be sampled in the same period of the year.

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CHAPTER 7

APPLICATIONS TO INDUSTRIAL HYGIENICS

7

TRACE ANALYSIS OF 3-HYDROXY BENZO[a]PYRENE IN URINE FOR THE BIOMONITORING OF PAH EXPOSURE

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ABSTRACT

Determination of benzo[a]pyrene (BaP) metabolites in urine can provide direct insight into the recent exposure to BaP, integrated over all uptake routes. In order to detect 3-hydroxy benzo[a]pyrene (3-OH BaP) in human urine after exposure to BaP at the workplace, extremely sensitive methods need to be developed. In this paper, a new extraction method is presented, and two laser-based fluorescence techniques are evaluated. Using HPLC with laser-induced fluorescence detection (HPLC-LIF), a detection limit of 8 ng/l was obtained. With laser-excited Shpol'skii spectrometry (LESS) after chemical derivatization, 3-OH BaP could even be detected at the 0.5 ng/l level.

In a pilot study, urine samples from coke oven workers and from occupationally non-exposed referents were analyzed. In the control samples, the average 3-OH BaP concentration was 8.3 ng/l; the 3-OH BaP levels were found to be highly correlated ($R^2 = 0.89$) with urinary 1-OH pyrene, a widely used biomarker for PAH exposure. Significantly enhanced 1-OH pyrene levels were measured in urine samples from coke oven workers, but in most samples a corresponding enhancement of 3-OH BaP was not observed. Possible explanations for this discrepancy are discussed.

INTRODUCTION

Exposure to polycyclic aromatic hydrocarbons (PAHs) is widely recognized as a serious health threat. Many PAHs are known or suspected carcinogens; benzo[a]pyrene (BaP) is the most thoroughly studied representative of this class of contaminants (Phillips, 1983). Human exposure to PAHs is often related to smoking, air pollution, or the consumption of charcoal broiled/smoked food products. Particularly high exposure levels are encountered in certain industries, where coal tar or derived products are produced or processed (Lindsted and Sollenberg, 1982). Epidemiological studies have revealed an increased incidence of respiratory cancers among coke oven workers (Lloyd, 1971). Protective measures should be taken in order to decrease these risks, and the exposure to PAHs should be thoroughly monitored.

Air sampling plays a central role in the identification of high-risk locations, but great care should be taken when air data are used to estimate the daily uptake. The PAH content in the air can vary considerably between different locations within a coke production plant (Jongeneelen et al., 1990). The numerous artefacts that may be introduced during sampling have been reviewed by Leinster and Evans (1986) and by Kirton and Crisp (1990). Most sampling devices are designed to collect PAHs bound to particulate matter, which results in an underestimation of the smaller PAHs (up to 4 rings), which are largely present in the vapor phase (Lesage et al., 1987; Kirton et al., 1991). Clearance of particle-bound PAHs from the respiratory tract (Sun et al., 1982), and the unknown contribution from dermal absorption are other factors that complicate the proper assessment of the actual PAH uptake at work.

In order to circumvent these difficulties, a biological monitoring approach may be chosen to determine the real internal dose, integrated in time and over all uptake routes. Upon uptake, PAHs are rapidly transformed into a number of hydroxylated metabolites. Some reactive intermediates have been shown to bind covalently to DNA or to proteins, but the largest fraction is rapidly excreted (Sun et al., 1982; Buckley and Lioy, 1992). Thus, analysis of PAH metabolites in excreta gives direct insight into the amount of PAHs actually absorbed. For practical reasons, urine is usually the preferred medium, although higher levels are usually encountered in faeces (Sun et al., 1982; Jacob et al., 1989).

Jongeneelen (1987) presented an analytical method, based on enzymatic hydrolysis, solid phase extraction (SPE) and high-performance liquid chromatography (HPLC) with fluorescence detection, for the determination of mono-hydroxy metabolites of pyrene, benz[a]anthracene, and BaP in urine. Compared to a reference population, enhanced levels of 1-hydroxy pyrene (1-OH pyrene) were found in urine samples from workers after handling creosote, but 3-OH benz[a]anthracene and 3-OH BaP could not be detected (reported limit of detection ca. 100 ng/l). Jongeneelen suggested the use of 1-OH pyrene as a biomarker for general PAH exposure, and demonstrated the practical applicability of that approach in a number of field surveys (Jongeneelen et al., 1988). Since then, the 1-OH pyrene assay has been widely adopted (Calderon, 1991; Boos et al., 1992; Buckley and Lioy, 1992).

The use of urinary 1-OH pyrene levels as an indication of the total PAH exposure is, of course, only valid if the uptake of pyrene is correlated to that of other PAHs, for instance that of the potent carcinogen BaP. Such a correlation would be supported by the experimental demonstration of a relation between the urinary excretion of 1-OH pyrene and that of other PAH metabolites, for instance that of 3-OH BaP, the major metabolite of BaP. This experimental verification would require extremely sensitive methods for the detection of urinary 3-OH BaP, since the fraction of 3-OH BaP excreted in urine is much smaller than that of smaller, more polar metabolites like 1-OH pyrene or hydroxy phenanthrenes (Grimmer et al., 1991). However, Jongeneelen and coworkers (1986) were able to demonstrate a correlation between 1-OH pyrene and 3-OH BaP concentrations in urine samples of dermatological patients after treatment with a coal tar ointment (extremely high exposure to PAHs). The 3-OH BaP concentrations were a factor of 2,500 lower than the 1-OH pyrene levels. In order to investigate whether a similar correlation can be found for occupationally exposed workers or for control persons, analytical methods will have to be developed that are sufficiently selective and sensitive to detect and quantitate 3-OH BaP at the low ng/l (ppt) level. Gas chromatographic methods are hampered by the low volatility of 3-OH BaP, even after derivatization (Krahn et al., 1987). Uziel et al. (1987) presented a fast synchronous fluorescence method for the quantitation of another BaP metabolite (7,8,9,10 tetrahydro tetrahydroxy BaP), but the detection limit of 25 µg/l is much too high for application to human exposure studies.

In this paper, the development and testing of two analytical approaches is described; both methods are based on a rigorous sample clean-up, followed by selective (laser) excitation and sensitive fluorescence detection. The first method uses a conventional HPLC chromatographic system; detection sensitivity is improved by using a laser as excitation source (laser-induced fluorescence, LIF). The second method is based on the Shpol'skii effect: the fluorescence spectra of planar, rigid compounds (like PAHs and some PAH derivatives) undergo a dramatic increase in resolution upon cooling in a suitable solvent that forms a regular crystal lattice at cryogenic temperatures (Shpol'skii, 1962; Wehry and Mamantov, 1981). The resulting intense, quasilinear spectra are very suitable for fingerprint identification (Mastenbroek et al., 1990), as well as for trace analysis in complex samples (Morel et al., 1991). Extra sensitivity and selectivity can be obtained with a laser excitation source, tuned to a specific narrow absorption transition of the analyte under investigation (laser excited Shpol'skii spectrometry, LESS) (D'Silva and Fassel, 1984). The applicability of LESS to the quantitative analysis of BaP metabolites in real samples was demonstrated by Ariese et al. (1993a; chapter 5).

Although the emphasis of the study was on method development, some interesting results from a first small-scale pilot study are also presented. 3-OH BaP was determined in 14 post-shift urine samples from coke oven workers. Control samples were obtained from occupationally non-exposed office personnel. 1-OH pyrene concentrations were measured in the same samples according to Jongeneelen et al.(1987). Associations between 3-OH BaP and 1-OH pyrene levels, and the validity of using the latter as a biomarker to estimate total PAH exposure, are discussed.

EXPERIMENTAL

Chemicals and safety

3-Hydroxy BaP was obtained from the NCI Chemical Carcinogen Repositories (MRI, Kansas City). Perylene d_{12} was purchased from Merck, Sharp & Dohme (Montreal, Canada). β-glucuronidase 30 U/ml with arylsulfatase activity 20 U/ml was supplied by Merck. n-Octane was obtained from Janssen Chimica; all other solvents were Baker analyzed grade. Methanol (Baker HPLC grade) and deionized water were distilled before use as eluent in the HPLC-LIF system. The derivatization of 3-OH BaP was carried out with strong base and methyl iodide (highly toxic); the use of a fume cupboard and protective clothing is recommended.

Origin of samples

End-of-third-shift urine samples from all top-side workers and door cleaners of the coke plant are regularly collected by the Dept. of Occupational Health and Safety. The type of job performed, any occurrence of process irregularities during the previous shifts, the use of airstream helmets (type AH4 RACAL Safety Ltd), as well as smoking habits are recorded. 1-OH pyrene levels are determined by HPLC-fluorescence following the procedure developed by Jongeneelen (1987). Fourteen samples, collected in autumn 1991, were randomly selected for 3-OH BaP determination (6 samples with HPLC-laser induced fluorescence and 8 samples with laser-excited Shpol'skii spectrometry)

Six control samples (3 Monday morning samples and 3 Friday afternoon samples) from occupationally non-exposed railroad office workers were randomly selected for 3-OH BaP determination. The samples, as well as creatinine and 1-OH pyrene data, were kindly provided by Dr Jongeneelen, Dept. of Toxicology, University of Nijmegen. Smoking habits were documented; no control person used special medication.

All samples and extracts were stored at -20 °C until further use.

Sample treatment

Urine extracts for 1-OH pyrene analysis were prepared according to Jongeneelen (1987). The sample treatment involved enzymatic hydrolysis of 10 ml of urine with β-glucuronidase/arylsulfatase at pH = 5 and 37 °C (16 hours overnight), followed by solid phase clean-up using Waters Sep-pak C18 cartridges. Trapped metabolites were eluted with 5 ml of methanol, evaporated to dryness in a gentle stream of nitrogen at 40 °C to remove traces of water, redissolved in 2 ml of methanol (sample enrichment factor 5), and centrifuged. The supernatant was transferred into air-tight vials; a small aliquot was taken for HPLC fluorescence determination of 1-OH pyrene. For 3-OH BaP determination, the extract needed further clean-up.

For HPLC-LIF analysis, 0.5 ml of the methanol extract was evaporated to dryness. After addition of 0.5 ml of water, 3-OH BaP was quantitatively extracted with 4 times 1 ml of n-hexane. For 3-OH BaP and 3-methoxy BaP, the recovery of repeated hexane extractions from aqueous phase was always > 97 % for spiked samples; no significant analyte concentrations

could be detected in the last extraction fraction or in the remaining water phase (< 1 %). The combined organic fractions were evaporated to dryness and redissolved in 0.5 ml of methanol (enrichment factor 5). Extraction with hexane was sometimes performed directly after the hydrolysis (see below).

For LESS analysis, 1.5 ml of the original methanol extract was evaporated to dryness. After addition of 0.5 ml of water, 3-OH BaP was quantitatively extracted with 4 times 1 ml of n-hexane, and the combined hexane fractions were concentrated to ca. 0.5 ml. For the derivatization of 3-OH BaP and other phenolic metabolites, the method of Weeks et al.(1990) was adopted. 2 mg of sodium hydride was put into a flask under dry nitrogen atmosphere and washed 3 times with pentane; after the addition of 1 ml of dry dimethyl sulfoxide (DMSO), the reaction mixture was stirred at 60 °C in a waterbath until the formation of H_2 bubbles had ceased. Upon cooling to room temperature, 100 µl of methyl iodide was added. The resulting derivatization mixture was added to the concentrated urine extract in hexane. The methylation of mono-hydroxy BaP metabolites was shown to proceed instantaneously and quantitatively (chapter 5; Ariese et al., 1993a). The derivatization reagent can be prepared in larger amounts to treat several extracts simultaneously. 3-Methoxy BaP was quantitatively extracted with 3 times 2 ml of hexane after the addition of 4 ml of water. The combined extracts were concentrated under nitrogen and the solvent was replaced with 375 µl of n-octane. The overall enrichment factor was 20. For quantitation of the Shpol'skii fluorescence signal, perdeuterated perylene was added (5 x 10^{-9} M).

In an alternative approach, the SPE step was omitted. After hydrolysis of 10 ml of urine, 3-OH BaP was quantitatively extracted with 4 times 10 ml of n-hexane or diethyl ether. Because of the large volumes involved, the extraction solvent should contain very low levels of fluorescent impurities and have a low boiling point. In the case of foam formation, a small table centrifuge was used to obtain a clear phase separation. The combined organic fractions were evaporated to dryness and redissolved in methanol (HPLC-LIF) or derivatized (LESS) as described above. Compared to liquid-liquid extraction after solid-phase extraction, the direct extraction from hydrolyzed urine saved a number of manipulations, and resulted in an equally effective clean-up with higher and more reliable yields (97± 2 % vs. 58 ±12 %).

HPLC with laser induced fluorescence detection

A schematic diagram of the HPLC-LIF setup is presented in Fig. 1. Reversed-phase separation was carried out using an LKB 2150 HPLC pump and a Chromspher C18 column (200 x 3 mm). The injection volume was 25 µl, the eluent was methanol/water 90:10 v/v, and the flow rate was 0.5 ml/min. The laboratory-made 4 mm square flowcell was made of Suprasil 1 quality quartz, with an internal round bore of 1.1 mm. The excitation source was a large-frame Innova 200 Argon ion laser operated in the multiline UV mode (1.3 Watt continuous at 352/364 nm), pumping a Coherent CR590 dye laser (Stilbene 3 in ethylene glycol). The dye laser (output 60 mW) was tuned to the 0-0 absorption transition of 3-OH BaP at 425 nm and focussed onto the

flow cell. Laser and flow cell were fitted on a low-vibration optical bench. Fluorescence was collected by a 600 μm i.d. quartz optical fiber put into the flowcell (Yeung and Sepaniak, 1980); the fibertip was positioned 1.5-2 mm above the laser beam. A Schott GG455 cut-off filter and a 466 nm interference filter (band pass 10 nm) were used to reject scattered laser light and define an emission window for 3-OH BaP. The detector was a Peltier cooled Philips XP 2020 Q photomultiplier tube, operated at 2200 V (PMT 1). The laser intensity was simultaneously monitored with a second optical fiber, collecting a fraction of the laser scattering from the flow cell (behind a frosted quartz plate to cancel out spatial fluctuations). The scattered light was attenuated to approximately the same level as the fluorescence detected by PMT 1, and measured by a second (identical) detector (PMT 2). The two PMT signals were digitized by a Stanford Research SR 400 photon counter (same time basis) and fed into a Macintosh personal computer with home-written data acquisition software. Ratioing could be carried out afterwards to correct for laser intensity fluctuations. The effluent from the flowcell was transferred on-line to a Perkin-Elmer LS-40 fluorescence detector, which allowed us to monitor the HPLC separation at other excitation/emission wavelengths or to compare the performance of the LIF-system with that of a state-of-the-art conventional fluorescence detector (van de Nesse et al., 1993).

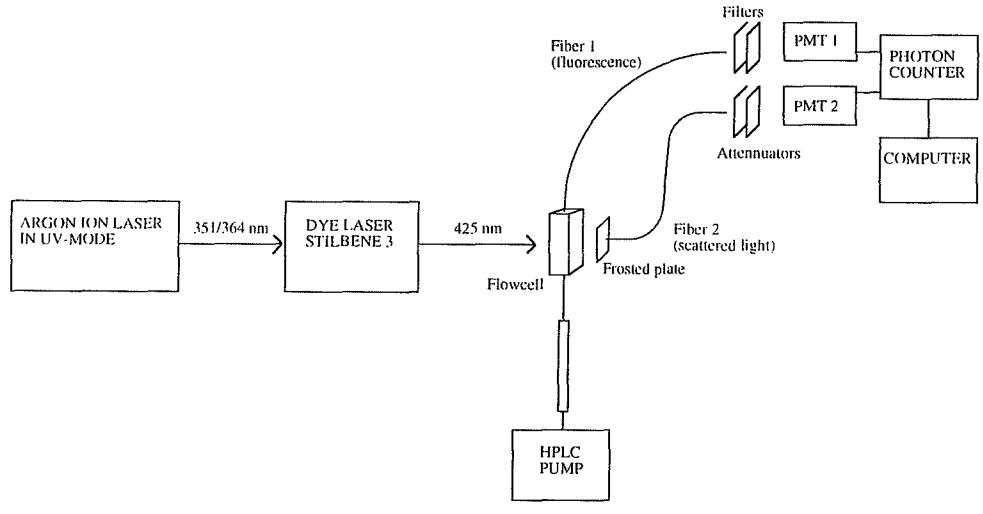

Fig. 1 Schematic diagram of the HPLC-laser-induced fluorescence apparatus. PMT = photo-multiplier tube. The effluent of the flow cell was transferred on-line to a conventional fluorescence detector.

Laser excited Shpol'skii spectrometry

A schematic diagram of the LESS setup is presented in Fig. 2. Shpol'skii spectra were recorded in n-octane polycrystalline matrices at 23 K. Four 10 µl samples could be cooled simultaneously in 45 minutes by means of a CTI cryogenics closed-cycle helium refrigerator. Laser excitation was provided by a Quantel YG580 frequency-doubled Nd:YAG laser (operated at 10 Hz, pulse width ca. 7 ns), pumping a Quantel TDL50 dye laser (oxazine 170). Frequency-mixing with the fundamental 1064 nm beam yielded a tunable range from 409-420 nm. 3-Methoxy benzo[a]pyrene was excited at 418.36 nm. The beam intensity was typically 1-3 mW per 3 mm^2 cross-section. Fluorescence was collected at a 20 ^{o} angle from the incident beam and focussed onto the entrance slit of a Spex 1877 triple monochromator (main grating 1200 grooves/mm; spectral resolution 0.1 nm). The detector was a Princeton Instruments (IRY 1024 GRB) intensified diode-array, covering a spectral range of 36 nm. The detector exposure time was typically 120 s per spectrum. Photochemical degradation of the analyte was less than 5 %, but prolonged irradiation (for instance during optimization of laser power or alignment) should be avoided. A straight calibration curve was obtained for 3-methoxy B[a]P in n-octane from 1 x 10^{-8} M down to 3 x 10^{-11} M ($R^2 > 0.999$). Perdeuterated perylene was added as internal standard at 5 x 10^{-9} M. For quantitation, the peak area of the 0-0 emission line of the analyte was ratioed with that of the internal standard. For time-resolved measurements a Princeton Instruments GF-100 pulser was used.

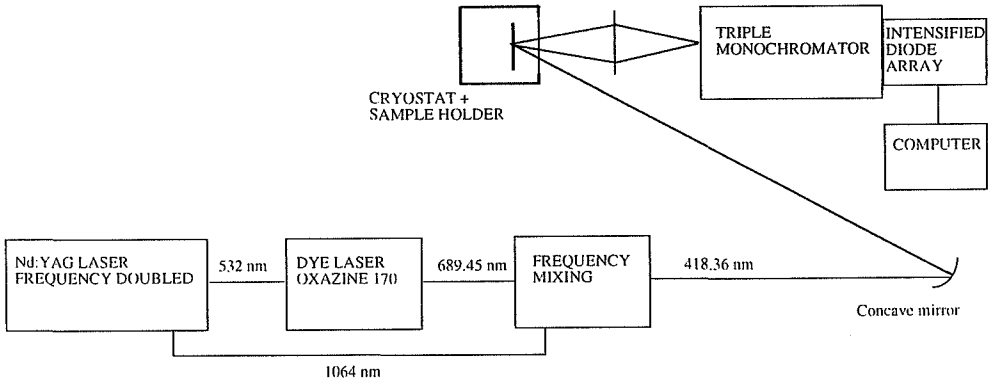

Fig. 2 Schematic diagram of the laser excited Shpol'skii spectrometry setup.

RESULTS AND DISCUSSION

Analytical performance of HPLC-LIF

First, the applicability of reversed phase HPLC with conventional fluorescence detection was evaluated. Jongeneelen (1986), using solid phase extraction and detector excitation/emission settings of 265/430 nm, reported a limit of detection (LOD) of 100 ng/l for 3-OH BaP in urine. Mathieu et al. (1985) obtained much lower detection limits using three successive extractions and a 2500-fold preconcentration. Rigorous clean-up should be the first step towards sensitivity improvement. The extract desorbed with methanol from a C18 cartridge is usually deeply colored and still contains a multitude of compounds that fluoresce when excited with short-wavelength UV light, resulting in detector overload at the onset of the chromatogram and in considerable background levels at longer retention times (see Fig. 3A). Normally, these interferences do not hamper the determination of 1-OH pyrene, which is present at sufficiently high levels (µg/l), even in control samples. However, the background precludes the detection of 3-OH BaP at the low ng/l level. Fluorescent interference should be dealt with first, before any significant improvements can be expected from concentration of the sample, higher excitation power, or better detector sensitivity. Furthermore, contrary to the satisfactory recoveries for 1-OH pyrene, the SPE recoveries for 3-OH BaP were rather low, which has also been noted by other researchers (Jongeneelen et al., 1985; Weyand et al., 1991).

Figure 3B illustrates the advantages of a more rigorous extraction method (liquid-liquid extraction with n-hexane). The hexane extract produces a low background, while no losses of the relatively lipophilic metabolites 1-OH pyrene and 3-OH BaP were observed when the extraction was carried out with at least twice an equal volume of hexane. Recoveries were better than 94 %.

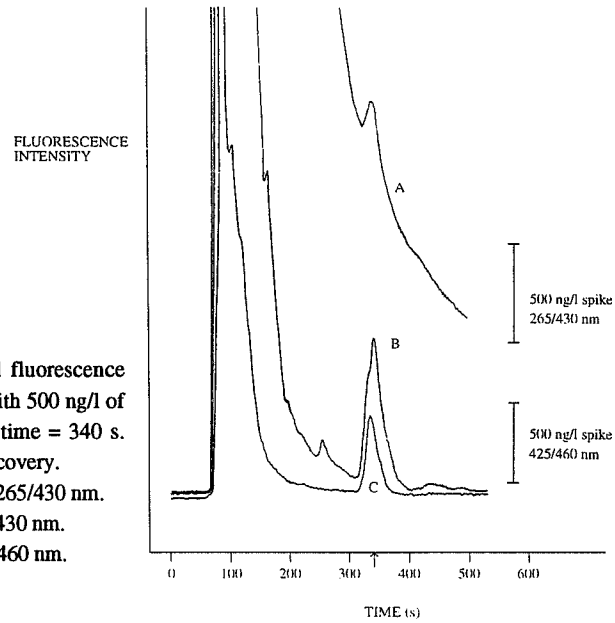

Fig. 3 HPLC chromatograms (conventional fluorescence detection) of a control urine sample, spiked with 500 ng/l of 3-OH BaP; enrichment factor = 5; retention time = 340 s. Vertical bars indicate peak height for 100 % recovery.
A: Solid phase extract; fluorescence detection 265/430 nm.
B: Hexane extract; fluorescence detection 265/430 nm.
C: Hexane extract; fluorescence detection 425/460 nm.

Figure 3C illustrates to what degree the remaining background fluorescence from the hexane extract (but also impurity emission from the eluent or from the walls of the flow cell), is further reduced when longer excitation/emission wavelengths are chosen. Although the extinction coefficient of 3-OH BaP is lower at 425 nm (16,800 $M^{-1}cm^{-1}$, compared to 41,600 $M^{-1}cm^{-1}$ at 265 nm; data provided by the NCI), and the emission is measured at a secondary maximum only, there is no significant loss of signal strength, since the spectral radiance of a typical xenon lamp is much higher at 425 nm than at 265 nm. The most important advantage, however, is that some interfering compounds, which are present in blank urine and coelute with 3-OH BaP, are not excited at longer wavelengths (compare Fig. 3B and 3C). Using the longest possible excitation wavelength (425 nm is the 0-0 absorption transition), a detection limit of 20 ng/l (2.5 pg) for 3-OH BaP in hexane-extracted urine samples was obtained. Nevertheless, the conventional HPLC/fluorescence system, with the Perkin Elmer LS40 detector, proved not sufficiently sensitive for most samples analyzed in this study.

After removal of most of the fluorescent interferences, the detectability will be limited by several instrumental factors (i.e. scattered light, lamp instability, detector dark current), by Raman scattering, and eventually by the statistical (quantum) nature of small photon fluxes (shot noise). The use of a laser excitation source may help overcome most of these problems. The advantages of laser-based detection are most obvious in the case of miniaturized separation systems (Dovichi, 1990), but LIF detection can also be applied to conventional-size HPLC. In that case, the better focussability of the laser is of minor importance, but the irradiance of the flow cell can be several orders of magnitudes higher than with conventional lamp excitation (Van de Nesse et al., 1993), resulting in a proportional increase in fluorescence intensity. Constant noise factors (i.e. detector dark current) will become negligible at higher signal intensities, but other factors, like Rayleigh and Raman scattering and impurity luminescence, will also increase proportionally with the laser power. The improvement in detector performance stems from the fact that, under shot noise limited conditions, an increase in irradiance of two orders of magnitude will result in a 100-fold increase in analyte signal and background intensity, but the noise of the background (equal to the square root of the detected number of photons) will increase only 10-fold. In principle, the signal-to-noise ratio of the analyte will continue to increase with the square root of the laser power, until other noise factors, usually laser instability or "flicker noise", become dominant (Ingle and Crouch, 1988).

An additional advantage of using a highly monochromatic light source is that the Raman spectrum of the eluent will become narrow-banded, which makes it easier to select a Raman-free window for the detection of the analyte fluorescence (Yeung and Sepaniak, 1980). With 425 nm laser excitation, 3-OH BaP emission can be measured between 456 and 480 nm without Raman interference from the methanol/water eluent. Impurity luminescence and Raman scattering from the walls of the flow cell are spatially filtered by the acceptance cone of the collecting fiber (Yeung and Sepaniak, 1980).

During the first experiments with laser excitation at 425 nm, no improvement was observed compared to the LS-40 detector, despite the increase in irradiance from ca. 40 μW to 60 mW (van de Nesse et al., 1993). Obviously, an additional noise source had been introduced. Since the fluorescence intensity is expressed as the number of photons counted per second (photon counting techniques were used to digitize the PMT signals), one can easily calculate the minimal noise level (shot noise), which should correspond to the square root of the signal height in the HPLC-chromatogram. However, the standard deviation of the eluent blank signal (root mean square noise) was significantly higher (see Fig. 4, upper trace), indicating the important contribution of other noise sources. Laser instability (flicker noise) was found to be the main cause of the observed noise level. A second detector was installed to monitor the laser intensity fluctuations. After ratioing the fluorescence signal point-by-point with the laser intensity, a three-fold decrease of the short-term noise level was observed (Fig. 4, lower trace). Thus, the limit of detection for 3-OH BaP in urine was improved to 8 ng/l (1.0 pg), which was, however, still not sufficiently low for most samples. Compared to conventional fluorescence detection, the improvement in detection limit is much less than expected on the basis of the increased excitation power, which has also been observed by van de Nesse and coworkers (1993). Finally, it must be emphasized that when a particular analyte in a chromatogram like Fig. 4 suffers from various interferences, unambiguous identification and proper quantitation is usually not possible.

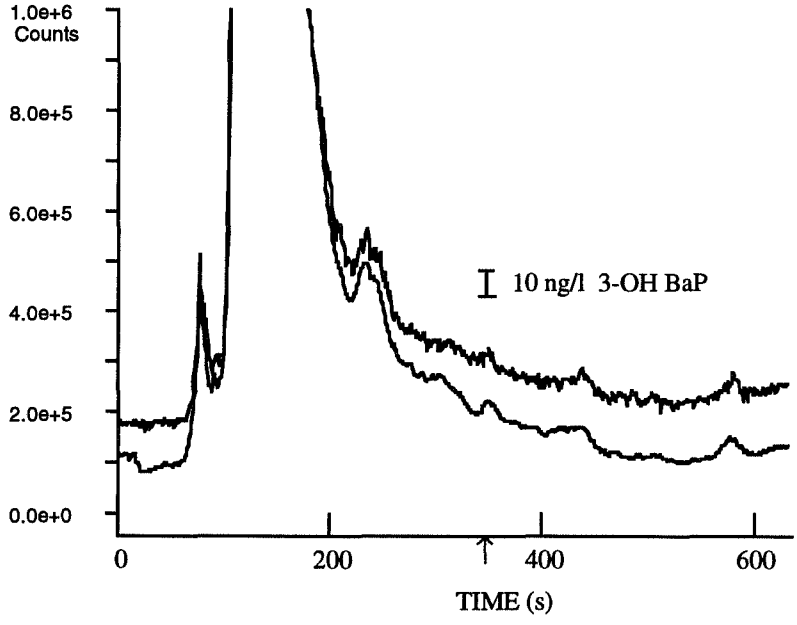

Fig. 4 HPLC-LIF chromatogram of urine sample W53. Upper trace: Original fluorescence signal from PMT 1. Lower trace: Fluorescence signal after correction for laser intensity fluctuations.

Analytical performance of laser excited Shpol'skii spectrometry

Theoretical, analytical, and instrumental aspects of the Shpol'skii method have been treated extensively by several authors (Shpol'skii, 1962; Wehry and Mamantov, 1981; Nakhimovsky, 1989); only a short overview will be given here. Shpol'skii spectrometry is a low-temperature technique, in which fluorescence spectra (sometimes phosphorescence or absorption spectra) are recorded after cooling the analyte to cryogenic temperatures in a suitable (poly)crystalline matrix. Matrix inhomogeneity, one of the main causes of spectral band-broadening at room-temperature, is strongly reduced when the analyte molecules occupy specific, well defined sites in the crystal lattice. The resulting fluorescence spectrum consists of a number of intense, sharp lines; the exact position of the 0-0 line in the particular matrix and the vibrational information provide a highly specific fingerprint of the analyte. Owing to the quasilinear character of the spectra (typical band widths 0.1 nm), spectral interference is strongly reduced compared to room temperature fluorescence measurements, and mixtures of fluorescent analytes can be analyzed without chromatographic separation. In a number of studies the applicability of the Shpol'skii method to the identification and trace determination of PAHs in complex samples has been demonstrated (Hofstraat et al., 1985; Morel et al., 1991).

Laser excitation may be used to increase the sensitivity of the measurement by increasing the irradiance of the sample, but an even more important advantage is the increase in selectivity. Under Shpol'skii conditions, the long-wavelength (S_1-S_0) part of the absorption spectrum consists of narrow lines (Nakhimovsky et al., 1989), and one can selectively excite one particular compound in a mixture by tuning the laser to a specific absorption transition (D'Silva and Fassel, 1984; Yang et al., 1981).

Most applications of the Shpol'skii technique concern parent PAHs in n-alkane matrices; few researchers have used the method for more polar compounds, like PAH metabolites (Khesina et al., 1975; Weeks et al., 1991; Ariese et al., 1993a). In a previous study, we found that mono-hydroxy BaP metabolites yield good Shpol'skii spectra in n-octane matrices, but the sensitivity was much lower than for the parent compound BaP (Ariese et al., 1993b). During the cooling process, the slightly polar metabolites tend to freeze out of the n-octane solution and form non-fluorescent aggregates. Transforming the phenolic -OH group into a less polar methoxy (OCH_3) group by means of a rapid derivatization reaction renders the metabolite more compatible with the n-octane matrix (Weeks et al., 1990; Ariese et al., 1993a), which results in a higher sensitivity and better reproducibility.

Fig. 5 (top) shows the high-resolution Shpol'skii spectrum of 3-methoxy BaP in n-octane at 23 K. Highest sensitivity and optimal selectivity was obtained when the laser was tuned to the vibronic absorption line at 418.36 nm. Other BaP metabolites are not excited at that wavelength (Ariese et al., 1993a; chapter 5). The laser line is rather close to the 0-0 emission transition, the most intense line of the fluorescence spectrum, but stray light was very effectively reduced by means of a triple monochromator. The intensified 1024 diode array could cover a sufficiently

large part of the spectrum at once (36 nm). Spectra were recorded by accumulating emission from a number of laser flashes until the required signal intensity was reached. In this configuration, laser instability does not add to the noise level of the spectrum, as all data points are equally affected. Since the Nd:YAG laser used was of the pulsed type, time-resolved detection could be used to discriminate between instantaneous scattering phenomena, analyte fluorescence (lifetime ca. 10 ns), and long-living background luminescence. Gated detection significantly reduced the detector dark current noise and allowed us to distinguish between quasilinear fluorescence and Raman lines, but in the case of real samples, the detectability was usually limited by short-living background luminescence from the extracts, and time-resolved detection proved of limited value.

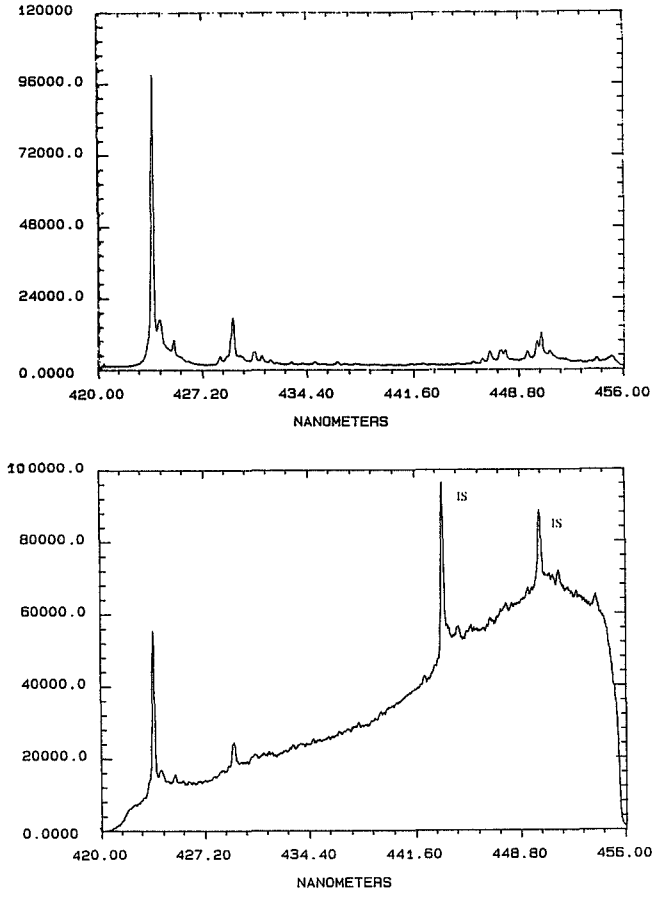

Fig. 5 Laser-excited Shpol'skii spectra of 3-methoxy BaP in n-octane at 23 K; λ_{exc} = 418.36 nm. Top: 5 x 10^{-8} M standard solution; detector exposure 4 s. Bottom: Urine extract W69 after chemical derivatization; detector exposure 120 s; IS = internal standard.

For the quantitation of Shpol'skii spectra, an internal standard must be added to the analytical sample in order to correct for long-term variation in laser intensity and optical alignment. The internal standard should give a quasilinear spectrum in the particular matrix and should not be present in the original sample or in the laboratory solvents used. For proper quantitation with LESS, the internal standard should be measured together with the analyte, which means that the internal standard should be excitable (to some extent) at the wavelength chosen for the analyte and at least one useful emission line should fall within the range covered by the multichannel detector. Perdeuterated perylene was found to meet these requirements.

The detection limit for 3-methoxy BaP in concentrated urine extracts was 4×10^{-11} M (0.1 pg), which corresponds to 0.5 ng/l in the original sample (enrichment factor 20). The sensitivity was sufficient for all urine samples encountered, even for controls. In a previous study, the repeatability of the method (4 replicates of sample treatment and determination) was found to be 16 %. (Ariese et al., 1993a). The LESS spectrum of 3-methoxy BaP in a urine extract from a cokeoven worker is presented in Fig. 5 (bottom spectrum). The exact position of the 0-0 emission line, together with the specificity of the excitation wavelength, provides a high degree of certainty about the identity of the compound, even when, close to the detection limit, vibronic emission lines are no longer detectable.

Determination of 3-OH BaP in control samples

Six control samples from occupationally non-exposed office personnel were analyzed with LESS. After derivatization with methyl iodide, 3-methoxy BaP could be detected in all samples. The LESS results are compiled in Table I, together with the 1-OH pyrene levels determined in the same samples by Jongeneelen with HPLC-fluorescence. As expected, Monday morning samples, being more concentrated, contained higher levels of both metabolites than Friday afternoon samples. After correction for creatinine content, there was a slight, but insignificant decrease during the working period, which was interpreted as an extra indication that occupational exposure to PAHs played no significant role for these referents. The average corrected excretion rates were 465 and 2.3 nmole/mole creatinine for 1-OH pyrene and 3-OH BaP, respectively, which corresponds to an average daily urinary excretion of 1620 ng 1-OH pyrene and 9.9 ng 3-OH BaP. The measured 1-OH pyrene values agree with data reported for larger reference populations (Jongeneelen et al., 1990.)

The 3-OH BaP concentrations detected in these samples were more than two orders of magnitude lower than the corresponding 1-OH pyrene concentrations; a correlation plot of the two metabolite levels is presented in Fig. 6 (open squares; $R^2 = 0.89$). Since the main sources of PAH uptake for the controls were diet and smoking, a certain degree of correlation is not unexpected. Total diet studies reveal that pyrene and BaP are usually present at more or less fixed ratios in PAH-containing food products; recent estimates of the average dietary daily intake of pyrene range from 600 ng (Pfannhauser, 1990) to 1600 ng (Vaessen et al., 1988). For BaP, daily ingested amounts of 50 ng (Pfannhauser, 1990), 114 ng (low-PAH diet; Buckley and Lioy,

1992) and ca. 200 ng (De Vos et al., 1990) have been reported. Similar ratios for pyrene and BaP are also found in cigarette smoke (Severson et al., 1976). Comparison of these intake estimates with the metabolite concentrations determined in our study shows that a substantial percentage of absorbed pyrene is excreted in urine as 1-OH pyrene. This agrees with the observation of Jongeneelen et al. (1990), who measured a urinary 1-OH pyrene excretion that was approximately equal to the calculated amount of inhaled pyrene, but contrasts sharply with the low urinary excretion rates measured in rats by Jacob et al. (1989). The fraction of ingested BaP that ends up as urinary 3-OH BaP is rather small, but still appears to be much higher in humans than in rats (Jongeneelen et al., 1985) and mice (Camus et al., 1984).

The average 1-OH pyrene/3-OH BaP ratio determined in this study in urine of non-exposed controls was ca. 200 (see Fig. 6). Jongeneelen et al. (1986) found a ratio of 2,500 in urine samples of dermatological patients after therapeutical treatment with a coal tar ointment. The pyrene/BaP ratio in the ointment was 2.4, which is lower than found in most food products. The (relatively) higher excretion of 3-OH BaP reported here is only partly explained by the better recovery of 3-OH BaP with the sample treatment used for our measurements. We suggest that the discrepancy may further be caused by a relatively slow dermal absorption rate for BaP, although differences in metabolite profiles between uninduced controls and PAH-induced patients cannot be excluded.

Table I 1-OH pyrene and 3-OH BaP concentrations in urine samples from controls.

Sample # [a]	Smoking [b]	1-OH pyrene ng/l [c]	3-OH BaP ng/l [d]
C40-Mo	+	1280	5.2
C43-Mo	+	2200	14.1
C45-Mo		3400	19.2
C39-Fr		400	2.3
C40-Fr	+	550	5.7
C41-Fr		1240	3.5

[a] Mo = Monday morning; Fr = Friday afternoon.

[b] + = smoker; - = non-smoker.

[c] Determined with HPLC/fluorescence.

[d] Determined with laser excited Shpol'skii spectrometry.

Of course it should be emphasized that the results presented here are the outcome of a small-scale pilot study. Nevertheless, the data fully support the basic assumption of the biological monitoring approach, that is, that the uptake of a more or less constant PAH-profile results in the excretion of a more or less constant PAH-metabolite profile. When the absorption of pyrene and that of more relevant compounds like BaP are expected to be correlated, as in the case of dietary exposure or smoking, the use of a single urinary metabolite (i.e. 1-OH pyrene) as a biomarker for the total PAH-exposure seems justified.

Determination of 3-OH BaP in urine samples from cokeoven workers

Fourteen post-third-shift urine samples were hydrolyzed, extracted, (for LESS also derivatized) and analyzed. Since the 1-OH pyrene concentrations in the samples were enhanced by approximately an order of magnitude compared to controls (Table II; in agreement with previous measurements of Jongeneelen et al., 1990), and since BaP had been detected at high levels in air samples taken in the personal breathing zone (Jongeneelen et al., 1990), 3-OH BaP levels in the 10-100 ng/l range were expected. Surprisingly, the 3-OH BaP concentrations in most samples were below the detection limit of the HPLC-LIF method (LOD = 8 ng/l). With laser-excited Shpol'skii spectrometry, 3-OH BaP could be detected in all samples, but in most cases the concentrations were indeed rather low (Table II). The 3-OH BaP and 1-OH pyrene levels determined in workers'urine are also incorporated into the correlation plot of Fig. 6; the dashed line indicates the ratio between the two metabolites as observed for the control population. Compared to the reference population, the 1-OH pyrene concentrations were clearly enhanced in all samples, but only in two cases (samples # W69 and W95), a corresponding enhancement of the 3-OH BaP level was actually observed. In most samples the 3-OH BaP concentrations were similar to those found for controls.

Since the whole concept of biological monitoring is based on the assumption that the excretion of PAH metabolites is related to the actual uptake, we conclude that most workers from the cokeoven battery monitored in this study had been exposed to high levels of pyrene, but not to similarly enhanced levels of BaP. A possible explanation for this discrepancy could be a contribution from skin exposure: dermal absorption could be more efficient for pyrene than for BaP. Skin exposure could contribute significantly to the total PAH uptake at a coke plant, but is difficult to quantitate (F.J. Jongeneelen, personal comm.).

One might suggest, however, that the apparently low exposure to BaP could also be attributed to the fact that most workers use an air stream helmet during work hours. The helmets contain a particle filter, that very effectively prevents the inhalation of BaP (which exists almost exclusively in the particulate phase (Lesage et a., 1987). Pyrene, on the other hand, is considerably more volatile and a large fraction can be present in the gaseous phase at the typical ambient temperatures encountered around coke ovens (Lesage et al., 1987). Furthermore, particle-bound pyrene, after having been trapped on the filter, could evaporate again and be taken along by the air stream drawn through the filter (Leinster and Evans, 1986). Thus, workers

Table II 1-OH pyrene and 3-OH BaP concentrations in urine samples from cokeoven workers.

Sample #	Smoking[a]	Helmet use[b]	1-OH pyrene ng/l [c]	3-OH BaP ng/l
W 1	+	+	14,000	< 8 [d]
W 2	+	+/-	19,000	3.0 [e]
W24		+	8,000	1.7 [e]
W25		+/-	11,000	<8 [d]
W29	+	+	13,000	<8 [d]
W39[f]		+	10,000	7.6 [e]
W53	+	+	10,000	9 [d]
W58		+/-	19,000	<8 [d]
W61[f]	+	+	16,000	2.0 [e]
W69	+	+/-	4,000	39 [e]
W76	+		10,000	<8 [d]
W83[f]	+		56,000	4.0 [e]
W89[f]	+	+	20,000	2.0 [e]
W95[f]			17,000	80 [e]

[a] + = smoker; - = non-smoker.

[b] Use of a protective air stream helmet at work; + = always; +/- = sometimes; - = never.

[c] Determined with HPLC/fluorescence.

[d] Determined with HPLC/laser induced fluorescence.

[e] Determined with laser excited Shpol'skii spectrometry.

[f] Process irregularity encountered during shift.

wearing particle-selective protection may still be exposed to relatively high levels of pyrene (and other volatile PAHs), owing to the same phenomena that cause sampling artefacts when airborne PAHs are collected with a particle filter. Jongeneelen and coworkers (1990), in a large-scale survey of PAH exposure levels at the same coke plant, observed that the use of an air stream helmet reduced the urinary 1-OH pyrene levels by approximately a factor of 2. Although the number of samples analyzed in this first explorative study is rather small, the data presented here suggest that the degree of protection against heavier PAHs like BaP is much better. For most workers, the total uptake of BaP seems not enhanced compared to the reference group. The two urine samples exhibiting high 3-OH BaP levels were collected (incidentally ?) from two workers who only sometimes or never wear an air stream mask. The metabolite concentrations determined in samples W76 and W83 are not explained by this hypothesis. The efficiency of particle filters against PAHs of different volatility and at different temperatures deserves further investigation.

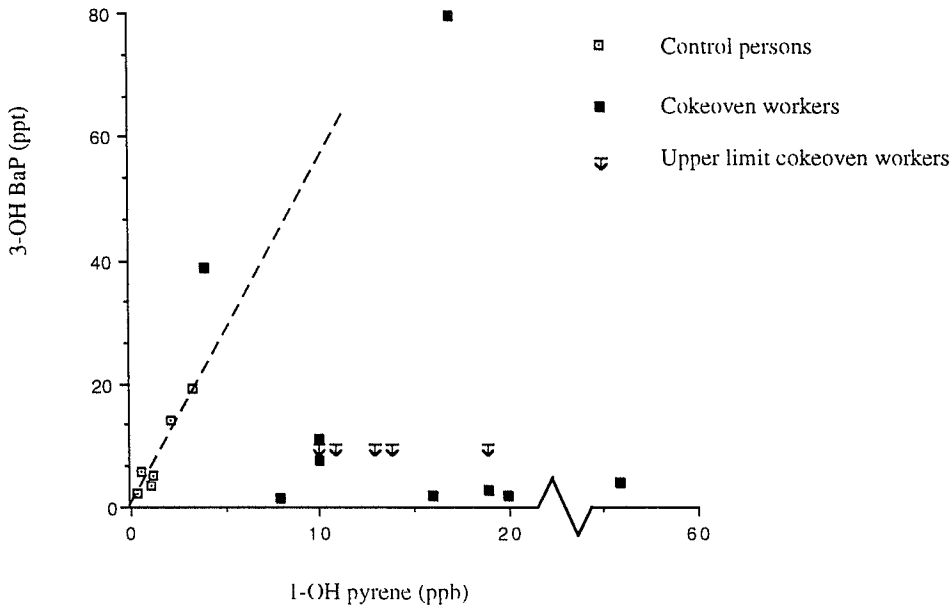

Fig. 6 **Correlation plot of 3-OH BaP against 1-OH pyrene levels in urine samples of controls and of cokeoven workers. Upper limits are indicated for those samples where the concentration was below the detection limit of the HPLC-LIF system. The dashed line indicates the average metabolite ratio found for the referents.**

SUMMARY AND CONCLUSIONS

Liquid-liquid extraction of 3-OH BaP from urine samples with n-hexane or diethyl ether resulted in a better clean-up with higher yields than solid-phase extraction with C18 cartridges. Interferences were further reduced by employing more selective long-wavelength excitation at 425 nm. For HPLC with conventional fluorescence detection, the LOD for urinary 3-OH BaP was 20 ng/l, which is probably too high for practical application to human exposure studies.

Detection sensitivity was improved with laser excitation at 425 nm, but only after correction for laser intensity fluctuations. Although 3-OH BaP could not be detected in most samples (upper limit 8 ng/l), the HPLC-LIF results still served as an important confirmation of the generally low values determined with LESS.

With laser-excited Shpol'skii spectrometry after chemical derivatization, 3-OH BaP was detected in all samples, including controls; the LOD was as low as 0.5 ng/l. Furthermore, compounds were identified with a high degree of certainty.

The 3-OH BaP concentrations in control urines were correlated with the 1-OH pyrene levels ($R^2 = 0.89$). These results support the routine use of the latter compound as a biomarker for the determination of the extent of exposure to a well-established and more or less constant PAH profile.

In urine samples from coke oven workers, the generally high 1-OH pyrene levels were only in two cases accompanied by an enhanced 3-OH BaP concentration. Apparently, most workers had been exposed to high pyrene levels, but not to BaP; some tentative explanations for the reduced exposure to heavier PAHs are discussed. In cases like this, the 1-OH pyrene assay leads to an overestimation of the exposure to larger PAHs.

We believe that the use of 1-OH pyrene as a first estimate of total PAH-exposure is certainly very useful. The method does not require overly sophisticated instrumentation, and pyrene is usually one of the most predominant PAHs under most working conditions. Whenever there are reasons to suspect a significantly different degree of exposure to other PAHs, more specific methods should be used to assess the uptake of toxicologically more relevant compounds like BaP. Thus far, only laser-excited Shpol'skii spectrometry (LESS) appears to offer the required sensitivity and selectivity for that purpose.

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CHAPTER 8

GENERAL CONCLUSIONS AND FUTURE DEVELOPMENTS

8
GENERAL CONCLUSIONS AND FUTURE DEVELOPMENTS

Shpol'skii spectroscopy of parent PAHs has shown to be applicable to various types of samples of ecotoxocological interest, like sediments or biota extracts. Because of the excellent identification capacities of the technique, it can be used in a qualitative way for the identification of unknown compounds and for the assessment of HPLC peak purity. Application of such a reference procedure with this highly specific technique for each type of sample would greatly improve the quality of routine HPLC measurements.

Considering the potential of Shpol'skii spectrometry as a quantitative analytical technique, a point questioned in the literature, it has been shown that accuracy and precision of the method are fully adequate if care is taken to assure reproducible sample preparation and cooling procedures, and if a proper internal standard is employed. The application of Shpol'skii spectrometry as an extra independent analytical technique will be especially useful in case large discrepancies are observed between analytical results and reference values or between analytical results obtained with different (chromatographic) methods.

Conventional Shpol'skii spectrometry can also be employed to the biological monitoring of PAH exposure (accumulation of parent PAHs), using an extraction and cleanup procedure similar to that routinely applied to HPLC analysis. It was demonstrated that Shpol'skii measurements in crude, lipid-rich extracts are also possible, but matrix distortions have to be accounted for. For such complex analytical problems, laser excited Shpol'skii spectrometry (LESS) is particularly useful.

The LESS method was also applied to the trace analysis of mono-hydroxy benzo[a]pyrene metabolites (after chemical derivatization of the phenolic groups), in order to assess exposure of fish to benzo[a]pyrene The uptake of this potent carcinogen was found to be correlated with the uptake of pyrene. 1-Hydroxy pyrene, the major metabolite of the latter, is easily determined in fish bile samples. The sensitivity of synchronous fluorescence spectrometry (SFS) for this marker metabolite was sufficient for most field applications.

The supreme sensitivity and specificity of LESS was also shown to be potentially useful in industrial toxicology, as it allows to assess the uptake of PAHs at work by trace determination of PAH metabolites in urine

Contrary to the determination of parent PAHs, which is routinely carried out by numerous laboratories in many different types of (environmental) samples, few researchers have apparently realized the usefulness of measuring PAH metabolites in excreta for monitoring purposes. In higher species with a high metabolic activity, such as fish or mammals, the bulk of a particular PAH absorbed by the body is rapidly detoxified and excreted. Since it is believed that PAHs need to be metabolized before any genotoxic effects can occur, it is the magnitude of this flux that eventually determines the actual exposure of an organism to PAHs. Determination of metabolites

provides a direct measure of the recent uptake, integrated over all uptake routes. Quantitation of the actually absorbed dose can bridge the gap between contaminant levels in the environment and possible effects in a particular population. On the other hand, the measurement of low steady-state body burdens of parent PAHs seems of limited value and sometimes even shows a negative correlation with environmental contaminant levels (Van der Oost et al., 1993).

Comparison of PAH metabolite concentrations with parent PAH levels in the environment is a straightforward method to assess the bioavailability of the particular compound. In this study it was found that the uptake of pyrene by fish from PAH-containing sediment was more than two orders of magnitude faster than that of benzo[a]pyrene (BaP). Similar differences in PAH uptake rate between small, relatively well soluble PAHs and larger, less mobile aromatics have been reported by Krahn et al. (1987) and by Landrum (1989) for other aquatic species. Aquatic toxicologists should study the carcinogenic properties of smaller PAHs in more detail. At present, most toxicological experiments with fish are being carried out with BaP as a model carcinogen. Owing to the large differences in bioavailability in the aquatic environment, it should be investigated whether smaller PAHs with two, three, or four rings, which are believed to possess only relatively low carcinogenic potencies, but are absorbed by fish at much higher rates than BaP, could not contribute significantly to the overall PAH-related genotoxic risks. The argument that not only BaP is responsible for adduct formation in fish populations is supported by the observation that adduct patterns obtained with the ^{32}P postlabeling assay from flounder and eel liver DNA revealed a variety of different adducts, which could not be attributed to known BaP-DNA adducts. Very sensitive and highly specific methods, probably combining enzymatic, chromatographic and spectroscopic selectivity, need to be developed for the identification of these adducts.

Krahn and coworkers (1987) reported that the incidence of hepatic neoplasia in fish populations correlated better with metabolite levels in the bile of these fish than with PAH levels in the sediments. As discussed in section 6.2, sediment data can be misleading when determined in the vicinity of a point source, or if the major part of the total exposure stems from suspended matter (fast streaming systems). Furthermore, there are several ways of measuring and expressing contaminant levels in sediments: determination in total sediment or in fine-grained material only, correction for organic carbon content, correction for grain size distribution (Klamer et al., 1990). Mesocosm experiments are presently being carried out to study which sediment characterization method is the best predictor for PAH uptake by fish. From an ecotoxicological point of view, measuring the PAH uptake by the target organism, as determined by biological monitoring, is more relevant than measuring environmental contaminant levels.

In industrial toxicology, metabolite determination has become more widely accepted as a monitoring tool for the assessment of occupational exposure to PAHs. The use of 1-hydroxy pyrene as an indicator of total PAH exposure, as suggested by Jongeneelen and coworkers (1988), seems a useful approach. At this point it appears important to determine the transformation efficiency of pyrene from diet (and smoking) into urinary metabolites, as these are

the major factors determining the 1-hydroxy pyrene background levels. The same could be carried out for benzo[a]pyrene, now that laser excited Shpol'skii spectrometry has shown to offer the required sensitivity. There are indications (see chapter 7) that for both compounds the fraction that ends up in the urine is much larger for humans than was estimated on the basis of laboratory experiments with rodents.

Urinary metabolite levels reflect the total PAH absorption, summed over all uptake routes, which means that this biomonitoring approach can only be applied if the exposure at the workplace is of the same order of magnitude or exceeds that of the (dietary) background. If the amount of inhaled PAH is smaller than the daily uptake from food, but still toxicologically relevant because of the higher susceptibility of the lungs, then more route-specific monitoring strategies must be followed.

The use of a single marker metabolite to assess exposure to total PAH at the workplace is of course only valid if the PAH profile is roughly constant. Most epidemiological data refer to situations in which people were exposed to a typical combustion-PAH profile. Under certain conditions the uptake profile may be very different, for instance when handling coal tar distillation fractions, when PAH exposure results from evaporation of smaller PAHs during paving/roofing, or when protective devices are used. In these situations, the contribution of more important carcinogens to the total uptake should be studied employing a specific method like LESS.

In general, we can conclude that PAH pollution levels are regularly monitored in the environment, and that a number of PAHs have shown carcinogenic activity when administered in high doses. On the other hand, we still know very little about the carcinogenic potency of mixtures of contaminants under real-world conditions at the low doses usually encountered in the field. In other words, much money and effort is being invested into collecting data on PAH levels, but the present knowledge is insufficient to judge whether the measured values should be considered as serious or not. In order to find an answer to these questions, the determination of PAH metabolites (and of PAH-specific effects at the biochemical level such as adduct formation), is expected to provide some important clues, especially if the methods are sufficiently sensitive and practical for field monitoring. The development of new, more sensitive, more specific, or more rapid methods is a challenge to analytical chemists. Considering the strong fluorescence of most PAHs and PAH derivatives, fluorescence-based techniques will undoubtedly continue to play a key role.

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SUMMARY

In this thesis, Shpol'skii spectrometry and synchronous fluorescence spectrometry are evaluated as for their applicability to the analysis of polycyclic aromatic hydrocarbons (PAHs) and PAH metabolites. PAHs are formed during the incomplete combustion of fossil fuels or organic matter, and can also be present in petrochemical products. After uptake by an organism, some PAH congeners can be metabolized into potent mutagens; the high incidence of liver tumors in certain fish populations or the enhanced death rate from lung cancer in certain industries are believed to be related to PAH exposure.

Apart from carrying out regular measurements of PAH concentrations in air, soils, sediments, water, and food, it is also important to investigate to what extent animals and humans actually absorb these chemicals. The latter approach is generally referred to as biological monitoring, and may involve measurement of steady-state accumulation levels of the parent compound in tissues (in case of slowly metabolizing species), or the determination of PAH metabolites in excreta samples, like urine, faeces, or gall-bladder bile. This method is particularly useful for higher species that rapidly metabolize PAHs, and can be used to assess the actual PAH flux through the body.

Most PAHs and PAH metabolites are strong fluorophores, and both techniques studied in this investigation are based on fluorescence detection. The first technique, the Shpol'skii method, is based on the fact that some compounds show narrow-banded fluorescence spectra upon cooling to cryogenic temperatures in a (poly)crystalline matrix. Highly specific spectra can be obtained for many PAHs and, as demonstrated in this thesis, also for some PAH metabolites using n-alkane matrices and temperatures < 30 K. These Shpol'skii spectra can serve as fingerprints for unambiguous, isomer-specific identification and allow the direct determination of PAHs in complex mixtures. Theoretical, instrumental, and analytical aspects of the Shpol'skii technique are described in chapter 2.

In chapter 3 the second technique, synchronous fluorescence spectrometry (SFS), is described. SFS is a room temperature technique in which fluorescence spectra are scanned with a constant difference between excitation- and emission wavelength. This method is less specific than Shpol'skii spectrometry, but can be used for rapid, low-cost screening.

In chapter 4 several applications of the Shpol'skii method to parent PAH analysis are described. For the study of section 4.1 a suspended matter sample was fractionated by means of reversed-phase HPLC, and the various PAH-containing fractions were frozen in n-hexane or n-octane. Low temperature Shpol'skii spectra were recorded for the identification of unknown peaks in the chromatogram, and to judge peak purity in a qualitative way.

The applicability of the Shpol'skii technique for quantitative measurements was investigated within the framework of an international intercomparison exercise for synthetic mixtures (section 4.2), and for sediment reference materials (section 4.3). After the main sources of error in sample treatment and spectroscopic analysis had been identified, the Shpol'skii technique was found to produce fully adequate quantitative results as compared to chromatographic analysis, and seems especially suitable as an independent reference method.

In section 4.4 the Shpol'skii method was shown to be applicable to extracts of mussel (*Mytilus edulis*) and tern (*Sterna hirundo*) tissue samples, after a sample pretreatment procedure commonly applied prior to HPLC analysis. Shpol'skii spectra could also be obtained in crude, lipid-rich extracts, but heavy dilution was necessary to reduce matrix absorption and to avoid serious distortion of the crystalline matrix. Nevertheless, pyrene, benzo[a]pyrene, and benzo[k] fluoranthene were directly determined in the crude extracts.

The sensitivity and selectivity of the Shpol'skii technique can be increased dramatically when a laser is used as excitation source. In chapter 4.5 the advantages of laser excited Shpol'skii spectrometry (LESS) are illustrated for the direct determination of PAHs in crude sediment- and tissue extracts. Laser beams are easily focussed on small sample volumes. Even more important is the increase in selectivity, which stems from the fact that under Shpol'skii conditions also the S_1-S_0 part of the absorption spectrum consists of narrow lines. Tuning the wavelength of the highly monochromatic laser system to the energy of a specific absorption transition thus results in the selective excitation of one particular analyte in a complex mixture. Time-resolved detection may be employed for extra selectivity and noise reduction.

The uptake of PAHs from the environment can also be assessed by measuring PAH metabolites in excreta. In fish, these detoxification products can be determined in bile fluid. In chapter 5 the Shpol'skii analysis of several metabolites of the potent carcinogen benzo[a]pyrene (BaP) is described. These compounds were determined in bile of flounder (*Platichthys flesus*) following injection with BaP, or after exposure in mesocosms to sediments with different contamination levels. For optimal compatibility with the low temperature n-octane matrix a derivatization reaction was employed to transform the hydroxy groups into less polar methoxy groups. Methylation was found to proceed fast and quantitatively for phenolic hydroxy groups, but not for dihydrodiol metabolites. Using enzymatic hydrolysis, methylation, and LESS detection, a quantitative protocol was developed for the trace determination of 3-hydroxy BaP in bile samples; the limit of detection (S/N = 3) was 5 ng/l. Bile of flounder exposed to moderately polluted harbor sediment from Rotterdam contained 40 times higher 3-hydroxy BaP levels than flounders exposed to a Wadden Sea sand bottom. Direct contact with the sediment and/or ingestion of particles appeared more important than uptake from the water phase or via the food chain.

In section 6.1 it is described how 1-hydroxy pyrene, a detoxification product of pyrene and a major PAH metabolite in fish bile samples, is very easily determined by means of synchronous

fluorescence spectrometry. As a result of differences in biological availability, small PAHs like pyrene are absorbed and excreted at a much higher rate than larger, more carcinogenic PAHs like BaP. Although pyrene is not believed to be very toxic, the extent of pyrene uptake could be used as a relative measure for the total PAH exposure, provided the PAH uptake profile is more or less constant. In flounder bile, the concentration of 1-hydroxy pyrene was found to be correlated with that of 3-hydroxy BaP. The 1-hydroxy pyrene concentrations were on the average 300-600 times higher. The SFS analysis is carried out in crude samples, only dilution is necessary. The detection limit is 10-20 ng/l, which is amply sufficient for most field applications.

In section 6.2, the simple SFS method was applied to a number of field surveys at the southern North Sea, and at some Dutch estuarine and freshwater locations. Usually the 1-hydroxy pyrene concentrations were similar in bile from different fish species captured at the same location, indicating the general applicability of the method. At the North Sea pyrene uptake levels were low at most locations, except for a narrow strip along the Dutch coast north of the mouth of the river Rhine, which suggests that river output of polluted sediment is the major contributor to the total (combustion-related) PAH exposure. Much higher 1-hydroxy pyrene levels were encountered in fish bile samples from the Western Scheldt, from the North Sea Canal, and from some freshwater locations in the vicinity of Amsterdam. The data on PAH uptake collected in this study could probably provide some important clues to explain the observed incidences of liver tumors in fish populations.

In certain industries, workers may be at an elevated risk if they are exposed to PAH-containing products or to elevated PAH levels in the workplace atmosphere. The actual uptake of PAHs can be studied by means of PAH metabolite analysis in urine, but most routine analytical methods are not sufficiently sensitive and/or selective for the determination of 3-hydroxy BaP in urine samples. In chapter 7 three techniques are evaluated: laser excited Shpol'skii spectrometry, and HPLC with conventional or laser induced fluorescence detection. Applying an improved extravtion method, the detection limits were 0.5 ng/l; 20 ng/l, and 8 ng/l, respectively. Only the LESS method proved sufficiently sensitive for application to human studies; even background levels could be determined in urine samples from occupationally non-exposed controls. In control urine samples the 3-hydroxy BaP levels were correlated to the (much higher) 1-hydroxy pyrene concentrations, which would justify the routine use of the latter as a marker metabolite for total PAH uptake. Urine samples from cokeoven workers contained enhanced 1-hydroxy pyrene levels, but only in two samples correspondingly enhanced 3-hydroxy BaP levels were found: in most cases the uptake of BaP appeared similar to that of controls. Possible explanations for this discrepancy are discussed.

In chapter 8 the major results obtained in this study are briefly summarized. Shpol'skii spectrometry, in particular when a tunable laser is employed for selective excitation, has been found to be very useful for the sensitive and selective determination of PAHs in complex samples. With a fast and practical derivatization reaction the Shpol'skii method can be extended to mono-hydroxy metabolites. For specific cases, like the determination of 1-hydroxy pyrene in fish bile, the rapid SFS method can be a fast and attractive alternative.

The uptake of PAHs from a polluted environment is a very complex process and depends on many poorly understood parameters. On the other hand, the biological monitoring approaches described in this thesis provide direct information on the actual uptake, integrated over all uptake routes. Combining PAH metabolite data with environmental PAH levels is a straightforward way to study biological availability and PAH uptake rates, which is necessary to estimate the magnitude of PAH-related risk for a given species in a given situation. In this respect, the spectroscopic techniques investigated in this study are very useful for both environmental and biological monitoring of PAH exposure.

SAMENVATTING

In dit proefschrift wordt het onderzoek beschreven naar de toepasbaarheid van twee spectroscopische meetmethoden, te weten Shpol'skii spectrometrie en synchrone fluorescentie spectrometrie, voor de (milieu)analyse van polycyclische aromatische koolwaterstoffen (PAKs) en PAK metabolieten. PAKs is een verzamelnaam voor een groep chemische verbindingen, bevattende tenminste twee gefuseerde aromatische (benzeen)ringen en opgebouwd uit de elementen koolstof en waterstof. Deze stoffen worden gevormd bij de onvolledige verbranding van fossiele brandstoffen of ander organisch materiaal (benzine, olie, hout, tabak), en komen tevens voor in de meeste aardolieprodukten. Van een aantal PAKs is bekend dat ze, na opname in het lichaam, kunnen worden omgezet tot mutagene verbindingen. Het verhoogd voorkomen van levertumoren bij vissen op bepaalde lokaties en van longkanker bij bepaalde beroepsgroepen wordt dan ook door sommige onderzoekers in verband gebracht met blootstelling aan deze stoffen in respectievelijk het aquatisch milieu of op de werkplek.

Om deze redenen worden PAKs als een belangrijke klasse milieukontaminanten beschouwd, en worden regelmatig metingen uitgevoerd om de concentraties van deze stoffen in lucht, water, bodem en voedsel te bepalen. Tevens is het belangrijk om te onderzoeken in hoeverre mens en dier deze stoffen ook daadwerkelijk opnemen. Dit laatste wordt biologische monitoring genoemd en kan bestaan uit het bepalen van in weefsels opgeslagen PAKs (bij organismen die PAKs slechts zeer langzaam omzetten) of uit het bepalen van de afbraakprodukten (metabolieten) van PAKs welke het lichaam via urine of faeces probeert te verwijderen. De laatste methode is met name geschikt voor hogere soorten met een goed ontwikkeld enzymsysteem en kan gebruikt worden om de PAK flux door het lichaam te bepalen.

De meeste PAKs en PAK metabolieten zijn intens fluorescerende stoffen, en van deze eigenschap kan in de chemische analyse goed gebruik gemaakt worden. In dit proefschrift worden twee op fluorescentie detectie gebaseerde technieken bestudeerd, die tot dusver weinig worden toegepast in de milieuanalyse, maar die buitengewoon geschikt lijken voor de bepaling van PAKs en PAK-metabolieten.

De eerste methode, de Shpol'skii techniek, maakt gebruik van het feit dat de vaak weinig informatieve, breedbandige fluorescentiespectra van sommige verbindingen kunnen overgaan in zeer specifieke lijnenspectra, wanneer deze stoffen worden afgekoeld tot zeer lage temperaturen in een oplosmiddel dat een regelmatig kristal vormt. De op deze wijze verkregen fluorescentiespectra kunnen dienen als vingerafdruk voor de identificatie van onbekende stoffen en zijn tevens geschikt voor de bepaling van complexe mengsels. In hoofdstuk 2 worden de theoretische en apparatuur-technische aspecten van de techniek behandeld, en wordt tevens beschreven voor welk type stoffen de methode het meest geschikt is en op welke wijze kwalitatieve en kwantitatieve metingen kunnen worden uitgevoerd.

In hoofdstuk 3 wordt een tweede techniek, synchrone fluorescentie spectroscopie (SFS) beschreven, die weliswaar minder gevoelig en minder specifiek is dan de Shpol'skii methode,

maar die toch goed bruikbaar is als een snelle, goedkope screeningsmethode voor de bepaling van fluorescerende stoffen die in wat hogere concentraties voorkomen.

In hoofdstuk 4 worden verscheidene toepassingen beschreven van de Shpol'skii techniek voor de bepaling van PAKs in diverse monsters. Voor het onderzoek in paragraaf 4.1 werd een zwevend stof monster uit de Westerschelde gescheiden met behulp van vloeistofchromatografie. Vervolgens werd een 21-tal fracties opgevangen en afgekoeld tot 28 K in een geschikte hexaan- of octaan matrix. Met behulp van Shpol'skii spectroscopie kon een groot aantal onbekende PAKs worden geïdentificeerd en kon een (kwalitatieve) uitspraak worden gedaan over piekzuiverheden in het chromatogram.

De geschiktheid van de Shpol'skii methode voor kwantitatieve PAK-bepaling werd voor academische oplossingen getest in het kader van een internationaal ringonderzoek (par. 4.2) en voor sedimentmonsters aan de hand van gecertificeerde referentiematerialen (par. 4.3). Nadat enkele praktische onvolkomenheden in het meetprotocol waren opgespoord en opgeheven, konden met de Shpol'skii methode uitstekende kwantitatieve metingen worden uitgevoerd.

De PAK-belasting in het aquatische milieu kan worden onderzocht door te bepalen in welke mate PAKs zijn opgeslagen in het vetweefsel van ter plaatse levende organismen. De analyse van PAKs in vetextracten is in de praktijk echter vaak buitengewoon moeilijk; in par. 4.4 werd onderzocht of de Shpol'skii methode wellicht een geschikt alternatief is. Goede resultaten werden verkregen in opgeschoonde (ontvette) extracten van mossel (*Mytylus edulis*) en visdief (*Sterna hirundo*). Bij de Shpol'skii analyse van pure vetextracten bleek echter dat de kristallijne matrix, welke van cruciaal belang is voor de methode, ernstig verstoord kan worden door hoge vetgehalten en dat een minimale verdunning noodzakelijk is. Uiteraard heeft verdunning een nadelige invloed op de gevoeligheid van de methode, maar toch kon een drietal PAKs direct, zonder enige vorm van monstervoorbewerking, in de vetextracten worden bepaald.

Zowel de gevoeligheid als de selektiviteit van de Shpol'skii techniek kan enorm worden verbeterd wanneer als lichtbron een laser wordt gebruikt in plaats van een lamp. De voordelen van laser geëxciteerde Shpol'skii spectroscopie (LESS) worden geïllustreerd in par. 4.5 aan de hand van PAK analyses in niet-opgewerkte sediment- en vogelvetextracten. De verbetering in gevoeligheid is een gevolg van het feit dat het vermogen van een laser zeer goed op een klein monstervolume kan worden gefocusseerd. Nog belangrijker is de toename in selektiviteit, welke bereikt kan worden doordat onder Shpol'skii condities het absorptiespectrum van PAKs ook uit smalle lijnen bestaat. Door nu de energie (de kleur) van het monochromatische laserlicht af te stemmen op de energie van een specifieke absorptieovergang kan bereikt worden dat in een complex mengsel slechts één verbinding zal gaan fluoresceren. Bij gebruik van een gepulste laser kan tijdsopgeloste detektie nog voor extra selektiviteitsverbetering en ruisonderdrukking zorgen.

De opname van in het milieu aanwezige PAKs kan tevens worden onderzocht aan de hand van PAK-afbraakprodukten (metabolieten). Bij vissen kunnen deze worden bepaald in de galvloeistof. Hoofdstuk 5 beschrijft de analyse van verschillende metabolieten van het uiterst carcinogene benz[a]pyreen (BaP) in de gal van bot (*Platichthys flesus*) na injectie met BaP of na

blootstelling aan vervuild havenslib respectievelijk Waddenzeezand. Om een optimale compatibiliteit met de octaan matrix te verkrijgen bleek het noodzakelijk om de hydroxygroepen eerst via een methyleringsreaktie om te zetten in methoxygroepen. Deze reaktie bleek snel en kwantitatief te verlopen voor (fenolische) mono-hydroxy metabolieten, maar minder geschikt te zijn voor (verzadigde) dihydrodiol metabolieten. Met behulp van enzymatische hydrolyse, methylering en LESS detectie, kon 3-hydroxy BaP kwantitatief worden bepaald in galmonsters; de detectielimiet bedraagt 5 ng/l. Botten blootgesteld aan Rotterdams havenslib (baggerklasse II) bleken veertigmaal zoveel BaP op te nemen als botten levend op een bodem van Waddenzeezand. Direkt kontakt met vervuild sediment of het inslikken daarvan bleek een belangrijkere opnameroute te zijn dan opname via de waterfase of via de voedselketen.

In par. 6.1 wordt beschreven hoe de concentratie 1-hydroxy pyreen, een metaboliet van pyreen, zeer eenvoudig en snel te bepalen is in gal van vissen met behulp van synchrone fluorescentie spectrometrie (SFS).Als gevolg van verschillen in biologische beschikbaarheid kunnen kleinere, beter oplosbare PAKs zoals pyreen veel sneller worden opgenomen door aquatische organismen dan grotere, meer carcinogene PAKs zoals BaP. Hoewel pyreen zelf niet zeer toxisch lijkt te zijn, zou de mate van opname van deze verbinding toch kunnen dienen als maat voor de algehele PAK blootstelling, wanneer de verhouding in opname tussen pyreen en die van meer toxische PAKs min of meer constant is. In gal van bot bleek de concentratie 1-hydroxy pyreen inderdaad gecorreleerd aan de concentratie 3-hydroxy BaP; de concentratie 1-hydroxy pyreen was gemiddeld een factor 300-600 hoger. Voor analyse behoeft het monster slechts verdund te worden; de detektielimiet bedraagt 10-20 ng/l, hetgeen ruim voldoende bleek voor de meeste veldtoepassingen.

De eenvoudige SFS methode werd toegepast om de PAK-opname door vissen te bepalen op diverse lokaties in de Noordzee, in de Nederlandse kustwateren en estuaria, en in enkele zoetwatergebieden rond Amsterdam (par. 6.2). Over het algemeen waren in galmonsters van verschillende vissoorten op dezelfde lokatie de 1-hydroxy pyreen gehalten vergelijkbaar, zodat de methode algemeen toepasbaar lijkt. Op de Noordzee concentreert de PAK-vervuiling zich in een smalle strook langs de Nederlandse kust; verder op zee werden veel lagere gehalten gevonden. Een sterk verhoogde PAK-opname werd waargenomen bij de monding van de Schelde, in het Noordzeekanaal, en op diverse lokaties rond Amsterdam. De gevonden PAK-opname patronen langs de kust kunnen een verklaring betekenen voor het in die gebieden voorkomen van levertumoren bij platvis.

PAKs kunnen ook een risico inhouden voor werknemers, die op het werk in aanraking komen met PAK-houdende produkten of blootstaan aan PAKs die bij diverse verbrandings-processen ontstaan. De opname kan worden vastgesteld door analyse van PAK metabolieten in de urine; voor de bepaling van 3-hydroxy BaP zijn de meeste huidige analytische methoden echter niet gevoelig genoeg. In hoofdstuk 7 worden drie analytische methoden getest: vloeistof chromatografie met conventionele resp. laser geïnduceerde fluorescentie detectie en laser geëxciteerde Shpol'skii spectrometrie. Na verbetering van de extractieprocedure waren de detektielimieten respektievelijk 20 ng/l, 8 ng/l en 0.5 ng/l. Alleen de LESS techniek bleek

voldoende gevoelig, en was zelfs geschikt voor de bepaling van een achtergrondwaarde bij niet-blootgestelde controlepersonen. Bij urinemonsters van controlepersonen bleek de concentratie 3-hydroxy BaP gecorreleerd aan de van 1-hydroxy pyreen, hetgeen het gebruik van de laatste als markerverbinding als schatting voor de totale PAK-opname ondersteunt. Bij urinemonsters van werknemers van een cokesfabriek werd verhoogde blootstelling aan pyreen vastgesteld, maar de concentratie 3-hydroxy BaP bleek slechts in twee gevallen verhoogd ten opzichte van de controlegroep. Enkele mogelijke verklaringen voor de niet of nauwelijks verhoogde opname van BaP, ondanks een duidelijk verhoogde blootstelling aan pyreen, worden besproken.

In hoofdstuk 8 tenslotte worden de belangrijkste conclusies van het onderzoek kort geresumeerd. Shpol'skii spectrometrie, in het bijzonder in combinatie met laser excitatie, is zeer geschikt gebleken voor de gevoelige en selektieve bepaling van PAKs en PAK-metabolieten in complexe monsters. In een aantal gevallen is synchrone fluorescentie spectroscopie bruikbaar als alternatieve, snelle screeningsmethode.

Aangezien in een vervuilde omgeving de opname van verschillende PAKs door mens of dier via diverse opnameroutes vaak zeer complex is, valt de werkelijke blootstelling aan PAKs slechts moeilijk in te schatten aan de hand van in de diverse milieucompartimenten gemeten PAK-gehalten. Daarentegen kan met een biologische monitoring benadering op een vrij direkte wijze de werkelijke, integrale blootstelling worden bepaald en wordt zo informatie verkregen omtrent biologische beschikbaarheden en opnamesnelheden van de diverse PAKs. Deze informatie is nodig voor een gefundeerde beoordeling van de risico's voor bepaalde soorten of individuen op een gegeven locatie. De in dit proefschrift onderzochte techieken kunnen een belangrijke rol spelen bij zowel de milieumonitoring als de biologische monitoring van PAK-blootstelling.

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